Seasonality of Bacterial Phylotypes Affected by Bottom-up and Top-down Control in Piburger See, Austria

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There is a theory which states that if ever anyone discovers exactly what the universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states that this has already happened.

Douglas N. Adams,

"The restaurant at the end of the world"

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Summary

Planktonic freshwater bacteria represent a fundamental compartment of lakes as they govern biogeochemical fluxes and play a crucial role in the aquatic food web. Seasonal changes in abundance, biomass, and activity status of bacteria seem to be triggered by changing nutrient regimes (bottom-up control) and grazing by heterotrophic protists (top-down control). The major aim of this PhD thesis was the exploration of these factors and the investigation of temporal and spatial fluctuations of different bacterial lineages in the oligo-mesotrophic Piburger See, Austria. The effects of increased or eliminated grazing pressure in combination with elevated or *in situ* nutrient concentrations, respectively, were studied in a laboratory- and a field experiment. *Betaproteobacteria* profited from high phosphorus concentrations, but were preferably ingested by heterotrophic flagellates. Other lineages, such as *Cytophaga-Flavobacteria* and *Alphaproteobacteria* appeared to be protected from size selective grazing by the formation of aggregates and filaments, whereas the small cell sizes of *Actinobacteria* served as a different strategy to escape predation. An extensive seasonal study at Piburger See showed that *Actinobacteria* – although numerically dominant – contributed marginally to total bacterial biomass, whereas rare *Cytophaga-Flavobacteria* – together with *Betaproteobacteria* – represented the bulk of the bacterial carbon pool. Seasonal fluctuations of *Actinobacteria* were found in the epilimnion, while *Betaproteobacteria* and *Cytophaga-Flavobacteria* represented maximum numbers and biomasses in the hypolimnion, concomitantly with oxygen depletion during summer stratification. Bacteria with active amino acid incorporation were mainly affiliated with *Actinobacteria* and *Betaproteobacteria*, while *Cytophaga-Flavobacteria* showed no affinity to the offered substrate. Moreover, we identified three lineages within *Betaproteobacteria* which accounted for most of betaproteobacterial diversity and occupied distinct spatial and ecophysiological niches in the lake. In general, the observed bottom-up and top-down factors had a pronounced impact on the competitive abilities of different bacterial lineages which resulted in shifts in the composition of the bacterial assemblage.

Zusammenfassung

Planktische Bakterien sind entscheidende Komponenten von Seen, da sie einen Grossteil der biogeochemischen Stoffflüsse steuern und eine wichtige Rolle im aquatischen Nahrungsnetz spielen. Eine saisonale Sukzession der Zusammensetzung, Biomasse und Aktivität dieser Bakterien scheint in Zusammenhang mit Schwankungen in der Nährstoffzufuhr ('bottom-up Faktoren') und Räuberdruck durch bakterivore Einzeller ('top-down Faktoren') zu stehen. Ziel dieser Doktorarbeit war es, diese Faktoren zu untersuchen und die saisonale und räumliche Verbreitung unterschiedlicher Bakteriengruppen im oligo-mesotrophen Piburger See, Österreich, zu studieren. In einem Labor- und einem Freilandexperiment wurden die Auswirkungen von erhöhtem bzw. vermindertem Räuberdruck in Verbindung mit erhöhter bzw. *in situ* Nährstoffkonzentration untersucht. Dabei wurde festgestellt, dass *Betaproteobacteria* zwar von hohen Phosphorkonzentrationen profitieren, aber auch bevorzugt von bakterivoren Flagellaten ingestiert werden. Andere Bakteriengruppen, wie z.B. *Cytophaga-Flavobacteria* oder *Alphaproteobacteria* schienen durch die Bildung von großen Aggregaten oder Filamenten einen gewissen Fraßschutz vor Einzellern zu genießen, während *Actinobacteria* durch ihre kleine Zellgrösse geschützt waren. Eine extensive saisonale Studie am Piburger See zeigte auf, dass *Actinobacteria*, obwohl sie zahlenmäßig dominierten, nur einen kleinen Teil zur bakteriellen Biomasse beitrugen, während seltene *Cytophaga-Flavobacteria* – neben *Betaproteobacteria* – zum Grossteil des bakteriellen Kohlenstoffes beitrugen. *Actinobacteria* zeigten saisonale Fluktuationen im Epilimnion, während *Betaproteobacteria* und *Cytophaga-Flavobacteria* maximale Zellzahlen und Biomassen im Hypolimnion zeitgleich mit der Sauerstoffzehrung während der Sommerstagnation des Sees aufwiesen. Aminosäuren wurden vor allem von *Actinobacteria* und *Betaproteobacteria* aktiv aufgenommen, während *Cytophaga-Flavobacteria* keine Inkorporation der angebotenen Substrate zeigten. Ferner konnten wir drei Populationen von *Betaproteobacteria* identifizieren, die für den Grossteil der beobachteten Diversität verantwortlich waren und unterschiedliche räumliche und ökophysiologische Nischen im See besetzten. Zusammenfassend kann gesagt werden, dass die untersuchten bottom-up und top-down Faktoren einen starken Einfluss auf die Konkurrenz zwischen verschiedenen Bakteriengruppen ausübten, was zu Verschiebungen in der Zusammensetzung des Bakterioplanktons führte.

1. Introduction

1.1. Seasonality of freshwater bacterioplankton

Bacteria belong to the – not only numerically, but also functionally - most important organisms in freshwater habitats and account, besides phytoplankton, for most of the biogeochemical fluxes in aquatic systems. Numerous studies were carried out regarding seasonal aspects of freshwater bacterioplankton (e.g. delGiorgio & Gasol 1995, Simon et al. 1998, Gasol & Duarte 2000). However, most surveys focused on bulk parameters such as DAPI direct counts or bacterial bulk activity measurements. Since the establishment of fluorescence *in situ* hybridization techniques (FISH, Amman et al. 1995, Alfreider et al. 1996), a closer look at the role of different phylogenetic lineages is now possible.

Thus, there is increasing knowledge about seasonal aspects of different freshwater bacterial clades studied from various lakes ranging from oligo- to eutrophic (e.g. Pernthaler et al. 1998, Klammer et al. 2002, Burkert et al. 2003, Zwisler et al. 2003, Hahn et al. 2005, Allgaier & Grossart 2006, Schauer et al. 2006, Wu & Hahn 2006a, 2006b). Several authors reported on distinct seasonal patterns of gram positive *Actinobacteria*, with one to two peaks in spring and autumn (Glöckner et al. 2000, Burkert et al. 2003, Allgaier & Grossart 2006). Maxima of filamentous *Sphingobacteria* and *Cytophaga-Flavobacteria* were found during the decay of a phytoplankton spring bloom in Mondsee (Schauer et al. 2006) and Lake Constance (Zwisler et al. 2003), respectively. Filamentous morphotypes were also detected in high numbers at times of elevated grazing pressure by bacterivorous flagellates, and were affiliated to *Alphaproteobacteria* (Nishimura & Nagata 2007) or *Sphingobacteria* (Jürgens & Stolpe 1995, Pernthaler et al. 2004). Pronounced seasonal patterns were observed for the species-like PnecB and PnecC subclusters of *Betaproteobacteria* in Mondsee, Lake Taihu, and a small humic pond. Their high abundances during summer (Hahn et al. 2005, Wu & Hahn 2006a, 2006b), were strongly correlated with water temperature.

However, most seasonal studies concentrated primarily on the surface layer of the studied lakes. Depending on the lake type, this approach probably does not reflect the entire situation, since stratified lakes are characterized by strong depth gradients of physicochemical parameters (e.g. temperature, oxygen, phosphorus,

nitrogen, ect.). Deep hypolimnetic water strata are often rich in nutrients such as dissolved phosphorus or ammonia. Therefore, higher abundances and biomasses of microorganisms can be expected in deeper water layers and above the sediment (Cole et al. 1993). Moreover, lakes with an oxygen-depleted hypolimnion can represent highly diverse ecological niches for specialized microorganisms such as fermenting bacteria, denitrifiers, methylotrophs, or autotrophic sulfur bacteria (Casamayor et al. 2000; Lehours et al. 2007). Certain bacteria from the oxic layers may also be capable of a facultative anaerobic metabolism (Alonso & Pernthaler 2005). Unfortunately, depth profiles are still rarely studied (e.g. Burkert et al. 2003, Wu & Hahn 2006a).

1.2. Factors governing freshwater bacteria

Besides abiotic factors such as water temperature and radiation, substrates and nutrients (bottom-up control, e.g., carbon, phosphorus), viral infection, and grazing activities by bacterivorous protists (top-down control) are assumed to act as the main forces shaping aquatic bacterial assemblages (Pace & Cole 1996). Additionally, competition for limiting substrates and nutrients within the entity *Bacteria* was identified as another very important factor governing freshwater bacteria (Šimek et al. 2006).

1.2.1. Top-down control

The impact of bacterivorous protists on planktonic bacteria was extensively studied in laboratory as well as field experiments (for review see Pernthaler 2005 and references therein). It is widely accepted that predation by size-selective protists can lead to changes in bacterial morphology, assemblage composition, and production (Hahn & Höfle 2001, Jürgens & Matz 2002).

Betaproteobacteria harbour phylotypes which are very sensitive to protistan grazing, i.e. the R-BT065 subcluster (Šimek et al. 2001, 2005, 2006, Jezbera et al. 2005, 2006) or the BET3-445 phylotype (Pernthaler et al. 2001) of the beta I subdivision. Bacteria of the R-BT065 subcluster, however, can compensate grazing induced cell losses by high cell specific activities and doubling times if nutrient supply (esp. phosphorus) is adequate (Šimek et al. 2005, 2006, Hornák et al. 2006).

Some strains of a subcluster of *Betaproteobacteria*, (the PnecC cluster, Hahn 2003) seem to range at the lower size-dependent uptake limit of heterotrophic

nanoflagellates (Boenigk et al., 2004). The same was found for a narrow lineage within the class *Actinobacteria* (the Ac1 phylotype, Pernthaler et al. 2001), which was enriched in the presence of the mixotrophic flagellate *Ochromonas* sp. Therefore, small cell size may represent a sufficient grazing defense strategy (Posch et al. 1999). A contrary mechanism for bacteria to escape protistan predation is enlargement to reach the upper uptake limit of size selective grazers. The formation of inedible filaments, microcolonies, flocs or aggregates is known for several bacterial groups such as *Cytophaga-Flavobacteria* and certain subdivisions of *Proteobacteria* (Hahn et al. 1999, 2000, 2004, Šimek et al. 1997, 2001). Lab experiments with isolated bacterial strains revealed filament and microcolony formation in the presence of protistan predators (Hahn et al. 1999, 2000, Matz et al. 2002, Corno 2006). The underlying mechanisms of this high phenotypic plasticity of these strains are not yet clear. Such morphological adaptations could be induced by excretory products of predators acting as 'infochemicals' or be a consequence of enhanced growth rate (Hahn et al. 1999, Corno & Jürgens 2006).

1.2.2. Bottom-up control

Bacteria are the main consumers of dissolved organic matter in freshwater habitats; thus, they play a vital role in the carbon cycle (Ducklow & Carlson 1992). The impact of limiting nutrients on the development of total bacterial numbers and bulk activity has been investigated in detail (e.g. Chrzanowski & Grover 2001, Carlsson & Caron 2001 and references therein). Although organic carbon is often regarded as most important limiting factor (Cole et al. 1988), several studies demonstrated that phosphorus and nitrogen limitation can be even more crucial for freshwater bacterial growth (Morris & Lewis 1992, Elser et al. 1995). In many cases, limitation seemed not to be restricted to a single nutrient, but co-limitation with respect to carbon and phosphorus availability could be detected (Morris & Lewis 1992, Schweitzer & Simon 1995). Moreover, bacteria have the potential to incorporate surplus phosphorus even at low ambient concentrations and are, therefore, considered as strong competitors with phytoplankton (Jürgens & Güde 1990, Rothhaupt & Güde 1992, Vadstein 2000, Mindl et al. 2005). Even grazing induced resistance mechanisms might be related to nutrient limitations. Chemostat experiments with mixed bacterial assemblages revealed that protistan grazing

favours filamentous bacteria only under phosphorus limitation but not under carbon limitation (Matz & Jürgens 2003).

1.2.3. Competition

Intraspecific competition probably plays an important but often underestimated role for the composition of bacterial assemblages. On one hand, some bacteria seemed to be more competitive in the uptake of limiting substrates and therefore could suppress bacteria from lineages with a lower affinity (Cottrell & Kirchman 2000a). On the other hand, grazing resistant bacteria have a competitive advantage at times of high grazing pressure. Due to the interplay of top-down and bottom-up factors, it is still unclear which factors drive more the competition within the entity *Bacteria*. However, some betaproteobacterial clades seem to be more efficient in taking up nutrients and can establish high numbers due to fast doubling times. This 'opportunistic' strategy was described for the R-BT subcluster of the beta I lineage (Šimek et al. 2001, 2005, Hornák et al. 2006) and for the beta II lineage (Burkert et al. 2003). Other freshwater and also marine representatives of this 'opportunistic life strategy' are located within *Gammaproteobacteria*, which have been easily enriched and cultivated (Eilers et al. 2000, Pinassi & Berman 2003). A contrasting life strategy was found for *Actinobacteria* present in high abundances in oligotrophic ecosystems such as high-mountain lakes (Glöckner et al. 2000, Warnecke et al. 2005). These bacteria were outcompeted by *Betaproteobacteria* in dilution cultures (Burkert et al. 2003).

1.3. Methods for studying bacterial phylotypes

Due to the lack of morphological traits suitable for discrimination and insufficient cultivation approaches, the identification of bacteria based on rRNA sequences was a breakthrough in microbial ecology (DeLong et al. 1989, Amann et al. 1990). Fluorescence *in situ* hybridization (FISH) represents a powerful tool to link phenotypic with genotypic information (Amann et al. 1995) without prior cultivation. The combination of FISH with cloning, sequencing, and probe design, i.e. the so-called full rRNA cycle (Amann et al. 1995), is widely used in studies of marine and freshwater bacteria. Up to now, investigations applying FISH were mainly focused on abundances but not on biomasses of *Proteobacteria*-groups and of *Cytophaga-Flavobacteria*. Recently, an improved protocol of fluorescence *in situ* hybridization

was described by combining FISH with catalyzed reporter deposition (CARD-FISH, Pernthaler et al. 2002, Sekar et al. 2003), resulting in a substantially brighter staining. The bright and persistent fluorescence intensity of CARD-FISH preparations allows the detection of even very minute cells and the application of image analysis systems for biomass determination within different bacterial lineages. As the conversion of organisms (numbers) to biomass (carbon) is necessary to compare different compartments of the food web, biovolume and biomass evaluations of bacteria from different phylogenetic lineages are essential to study carbon fluxes.

Another improvement was the combination of FISH with microautoradiography (Lee et al. 1999, Cottrell & Kirchman, 2000). Microautoradiography was developed in the late 1960s to analyse the metabolic activity of prokaryotes under *in situ* conditions by direct visualization of microorganisms with active substrate uptake within a complex - but unknown - community (Brock & Brock 1968). While microautoradiography on its own does not provide taxonomic data, the combination with FISH or CARD-FISH allows for the identification of the assemblage composition and the activity of bacteria at the same time (Lee et al. 1999, Teira et al. 2004).

1.4. Study site – Piburger See

Piburger See is a small, oligo-mesotrophic pre-alpine softwater lake situated in the Oetztaler Alps (Tyrol, Austria). Geographic and morphometric details are listed in Table 1. The catchment area is dominated by coniferous forest and the geological bedrock consists of crystalline rocks. The lake is usually covered with ice from early December to April and shows a short spring circulation after ice break, which is often only partially renewing the anaerobic deep water body. Thermal summer stratification is rapidly established with very warm surface temperatures (up to 25°C). The duration and extension of autumnal overturn is strongly affected by the weather conditions but holomixis is commonly detected.

Table 1. Geographic and morphometric characteristics of Piburger See.

Parameter	Value
Longitude	45°11'30'' N
Latitude	10°53' E
Altitude	913 m.a.s.l.
Catchment area	2.65 km^2
Surface area	0.134 km^2
Maximum length	0.8 km
Maximum width	0.3 km
Volume	1.8 x 10^6 m^3
Maximum depth	24.6 m
Mean depth	13.7 m
Shore line	1.9 km
Shoreline development	1.5
Water retention time	approx. 2 years
Stratification type	Dimictic
Trophic status	Oligo-mesotrophic

As many other lakes, Piburger See was strongly affected by anthropogenic induced eutrophication during the late 1960s. The installation of an 'Olszewski tube' (a deep water removal tube) in 1970 led to a remediation (Pechlaner 1979, Psenner et al. 1984). A marked and rapid improvement of the lake's oxygen regime was attributed to the selective removal of anoxic hypolimnetic water by the Olszewski tube. External restoration efforts such as the installation of a sewage treatment plant and the decreasing load of agricultural fertilizers further supported the re-oligotrophication of the lake. Since the late 1980s phosphorus concentrations decreased and the oxygen saturation of the hypolimnetic water body improved, and presently the lake has reached an oligo-mesotrophic status (Fig. 1).

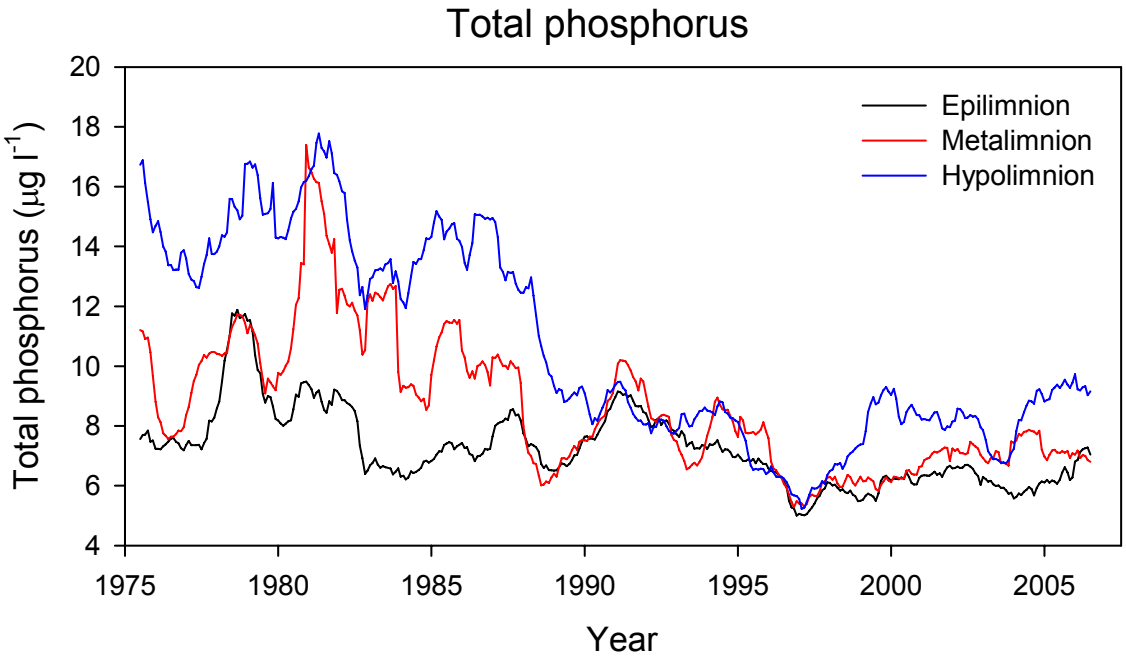

Figure 1: Changes in the concentrations of total phosphorus (µg l^{-1}) in Piburger See from 1975 to 2007 (Posch et al. unpublished).

The response of the lake to the restoration efforts was delayed about 20 years. In the first two decades after the implementation of the Olszewski tube a marked increase of the filamentous Cyanobacterium *Oscillatoria limosa* C. A. Agardh was observed, which contributed up to 40% to the total phytoplankton biovolume in 1974 (Rott 1976). Abundances of this species decreased since the late 1980s together with a massive reduction of phosphorus. Tolotti & Thies (2002) reported a shift of the summer phytoplankton species assemblage from green and filamentous blue green algae to small diatoms and coccal green algae since 1990. However, the phytoplankton assemblage is still undergoing progressive changes: In the years 2003-2005 a massive autumnal bloom of the diatom *Asterionella* sp. was observed, which decreased again in 2006 and 2007.

Piburger See is the subject of a detailed limnochemical monitoring program conducted by the Department of Limnology, Institute of Ecology, University of Innsbruck since the beginning of the 1970s (Tolotti & Thies 2002), with monthly sampling of the whole depth profile in a 3 m interval. Moreover, several projects (EU-projects such as REFLECT; CLIME, 5th Framework Program; EUROLIMPACS, 6th Framework Program) and numerous studies, diploma theses, PhD theses, and students' courses were performed at the lake, dealing with very different limnological topics. Sommaruga & Psenner (1995) reported on distinct patterns in seasonal

dynamics of bacteria (bulk parameters) and heterotrophic nanoflagellates, whereas Pernthaler and coworkers (1996a, 1996b) investigated top-down effects on bacteria grazed by protists. No detailed survey of bacterioplankton seasonality and the main factors shaping different phylogenetic lineages has been carried out until now.

2. Aims of this thesis

This PhD thesis was integrated in the project FWF ZFP175540 funded by the Austrian Science Fund. Therefore, some aims were already predetermined by the project proposal. However, new ideas and aims evolved throughout the whole period of the thesis.

The main goal of the project concerned the seasonal succession of bacterial phylotypes in Piburger See and the factors influencing bacteria from different clades. Simon et al. (1998) presented an idealized scheme of dynamics and control mechanisms of bacterioplankton in Lake Constance (Fig. 2), which was based on total bacterial abundances and production. It does not consider the composition of bacterial assemblages and therefore, deeper knowledge is needed about the main bacterial lineages which dominate freshwater ecosystems. We hypothesized that *Bacteria* could be split in several 'functional groups' or phylogenetic lineages which are differentially affected by top-down and bottom-up control and show varying responses to biotic and abiotic forces over the season.

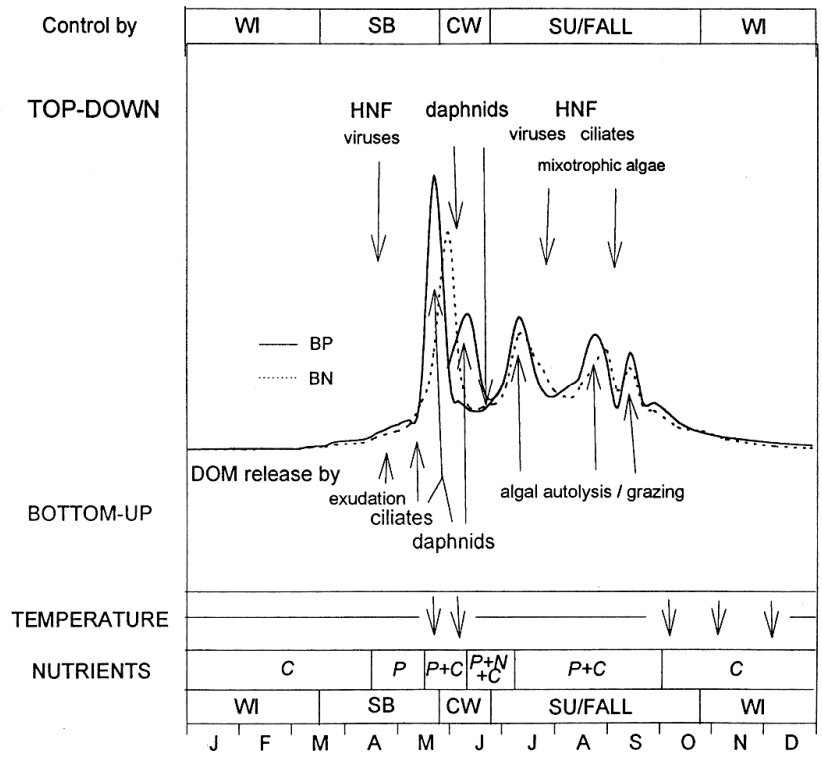

Figure 2: Idealized patterns of seasonal dynamics and control of bacterioplankton in Lake Constance (from Simon et al. 1998). WI: winter stratification; SB: phytoplankton spring bloom; CW: clear water phase, SU/FALL: summer and fall stratification; BP: bacterial production; BN: bacterial numbers; C: carbon; P: phosphorus; N: nitrogen.

Several specific questions were addressed:

- Is there a seasonality of bacteria from different phylogenetic lineages with respect to their abundances, biomasses and incorporation of radiolabeled amino acids?

- Are there spatial patterns (depth gradient) in the distribution of different bacterial lineages?

- How are bacteria affected by abiotic (chemical and physical) and biotic (phytoplankton and heterotrophic nanoflagellates) parameters and can they occupy different niches in the water column?

- Which bacterial populations profit from a sudden increase (or decrease) of protistan grazing and / or nutrient availability?

- Which bacterial lineages are most vulnerable against grazing by protists?

- Which bacterial lineages are limited by phosphorus?

3. Results and discussion

The results of this PhD thesis are presented in three already published research articles, two submitted manuscripts, and one manuscript in prep. These publications show the results of two experimental approaches (papers I – III) dealing with factors influencing bacteria from different lineages (top-down and bottom-up control), and an extensive seasonal study (manuscripts IV – VI). Additionally, some results of an - until now - unpublished data set are included presenting a generalized view on seasonal aspects of bacteria from different phylogenetic clades and their amino acid uptake activity and biomass distribution in Piburger See.

3.1. Part I: Factors influencing freshwater bacteria, i.e. bottom-up and top-down control, and competition

This part of my PhD thesis consists of three published articles. Most of the experimental work and data evaluation of the first article was already carried out during my diploma thesis. The article was written during the first period of my PhD study and thematically fits well to the other presented experiments. We studied the influence of top-down and bottom-up control shaping freshwater bacteria by two experimental setups. A short summary of each publication informs on the outcome of these studies.

paper I: Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community (Salcher et al. 2005)

In this study, a chemostat was used for examining the succession of microbial predator-prey interactions. The algae *Cryptomonas phaseolus* and its accompanying mixed bacterial assemblage were cultivated together in the first stage of a continuous cultivation system. Two bacterivorous predators were introduced to the second stage vessels, i.e. the mixotrophic nanoflagellate *Ochromonas* sp. and the heterotrophic ciliate *Cyclidium glaucoma*. Due to different levels of grazing, bacteria developed differential in these two setups. We found a succession not only of the bacterial assemblage composition, but also different phenotypic responses of bacteria within the same clades. This lab study provided us with background data about *Alphaproteobacteria*, *Betaproteobacteria*, and *Cytophaga-Flavobacteria* and their different response to high and medium levels of grazing. We observed that *Betaproteobacteria* were profiting in the absence of predators, while they were preferably ingested by both protists. The opposite effect was detected for *Alphaproteobacteria* and *Cytophaga-Flavobacteria*, which were enriched in the presence of the grazers. Different bacterial grazing defense mechanisms such as the formation of inedible filaments, microcolonies or aggregates were observed for bacteria affiliated to narrow taxonomic clades (i.e. the BET3-446 and the CAU663 clades within *Beta*- and *Alphaproteobacteria*, respectively), thus, exhibiting a high phenotypic plasticity.

paper II: Modulation of microbial predator-prey dynamics by phosphorus availability. Growth patterns and survival strategies of bacterial phylogenetic clades (Salcher et al. 2007)

We studied the *in situ* effects of bottom-up and top-down control of bacterioplankton in Piburger See in a size-fractionation and nutrient enrichment experiment. Two size fractions were generated via filtration of raw water samples over 5 or 0.8 µm filters to obtain variants with enhanced flagellate grazing due to the removal of larger predators (<5 µm) and predator-free variants (<0.8 µm), respectively. Duplicates of each fraction were incubated in bottles, bottles enriched with phosphorus, and dialysis tubes which allowed relatively free nutrient exchange with surrounding water. Enhanced grazing pressure resulted in a pronounced reduction of active bacteria, which was reflected in a decline of bacteria with high nucleic acid content and a lower detectability by FISH (probe EUB I-III). Moreover, a shift in bacterial assemblage composition was observed, with *Betaproteobacteria* being heavily grazed, whereas *Alphaproteobacteria* and *Cytophaga-Flavobacteria* seemed to be less affected. A shift in assemblage composition was also found after enrichment with phosphorus and even more pronounced in the dialysis tubes, where *Betaproteobacteria* became dominant and seemed to be more competitive in taking up surplus nutrients. In contrast, *Actinobacteria* did not respond to a higher amount of nutrients. First indications for different bacterial survival strategies at high grazing pressure obtained in the chemostat experiment (paper I) could be confirmed by this field study. In addition, nutrient availability was identified as another very important factor shaping bacterial assemblages.

paper III: Biomass reallocation within freshwater bacterioplankton induced by manipulating phosphorus availability and grazing (Posch et al. 2007)

This companion publication of paper II presents results of the same size-fractionation and nutrient enrichment experiment, but with a focus on bacterial stoichiometry and biomasses of different phylogenetic lineages. An increase of bacterial numbers, bulk activity, and biomasses in the bottle treatments enriched with phosphorus indicated bacterial phosphorus limitation. However, bacterial growth and biomasses increased even more in dialysis tube treatments, which led to speculations about co-limitation by carbon. The vast majority of increasing bacterial

biomass could be attributed to *Betaproteobacteria*, whereas *Actinobacteria* showed stable and - due to their small cell sizes - very low biomasses.

Summary of part I:

With these two experimental approaches we analysed the main shaping forces of freshwater bacterioplankton, i.e. grazing by flagellates and nutrient availability. Interestingly, we observed the same trends in the chemostat- and field experiments, i.e. the beta-subdivision of *Proteobacteria* was strongly affected by grazing, while members of the alpha-subdivision and *Cytophaga-Flavobacteria* showed higher grazing-resistance. Filamentous forms of the latter two lineages were found in the presence of predators and with high nutrient loads; therefore, the biomass of these groups was not as strongly reduced by grazing as their numbers. Moreover, we confirmed the importance of bottom-up factors for the establishment of dominant freshwater bacteria and their competitive abilities. *Betaproteobacteria* were identified as opportunistic competitors for phosphorus.

In paper II, we presented a schematic depiction of synergistic effects of bottom-up and top-down control inducing the dominance of bacterial clades in Piburger See (Fig. 3). This generalised view on bacteria from broad phylogenetic lineages summarizes the outcome of the three publications.

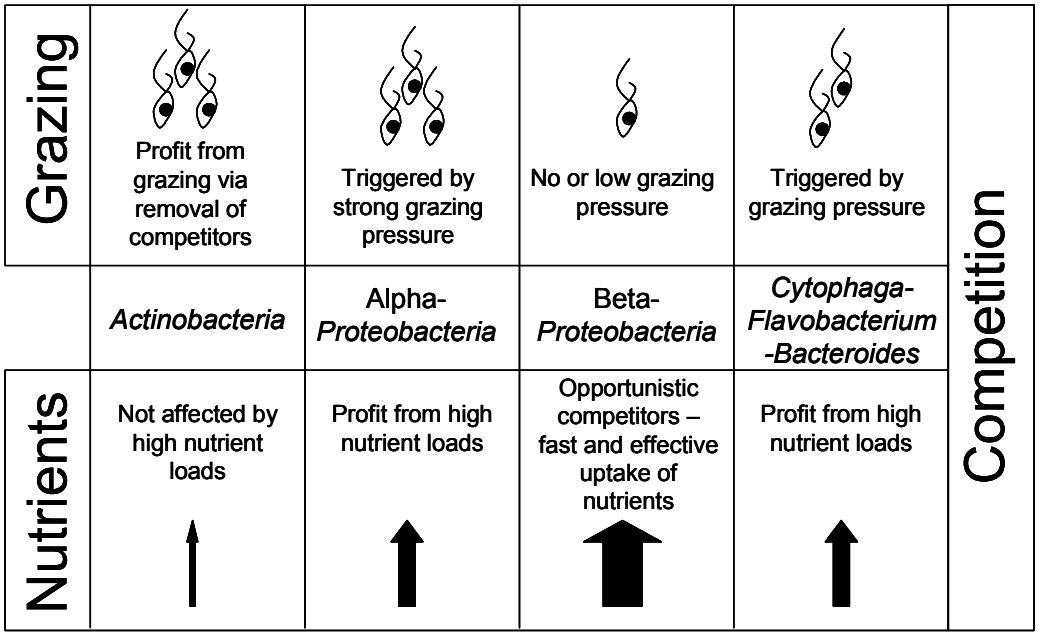

Figure 3: Schematic depiction of the synergistic effects of bottom-up and top-down factors and competition inducing the dominance of bacteria from different clades in Piburger See (from paper II, modified).

3.2. Part II: Seasonality of bacterial phylotypes in oligo-mesotrophic Piburger See, Austria

The second part of my PhD thesis consists of two submitted manuscripts, one paper in prep., and a short chapter containing some unpublished data. The first manuscript describes a new method for studying microbial morphotypes, whereas the other studies address seasonal and vertical changes of bacteria from different phylogenetic clades in respect to their abundances, biomass, and the ability to incorporate radiolabeled amino acids. The three manuscripts are summarized below to give a short overview on the main results.

manuscript IV: Morphotype specific contributions of hybridized cells to total bacterioplankton abundance and biomass as studied by image analysis. Positive relation of cell size to hybridization efficiency? (Posch et al. submitted to *Appl Environ Microbiol)*

Biomasses and morphotypes of hybridized bacteria were determined with a new technique based on image analysis techniques. While only <50% of DAPI-stained particles were found to hybridize with the general probe EUB I-III (specific for the domain *Bacteria*), the majority of bacterial biomass was detected (up to 89%). The total bacterial assemblage was mainly dominated by small cocci and rods; however, most of these morphotypes could not be detected by CARD-FISH. In contrast, larger morphologies showed much higher probability to be hybridized, which explained the high detection efficiency of biomass with CARD-FISH. Therefore, most bacterial carbon biomass could be analyzed, even at low numerical detection rates by the general probe EUB I-III. Moreover, we analysed morphotype distributions of three phylogenetic lineages. *Actinobacteria* were represented predominately by small rods and cocci, whereas *Cytophaga-Flavobacteria* formed mainly large rods and filaments. *Betaproteobacteria* showed the highest morphological variability. Thus, our new approach could help to elucidate the relationship between geno- and morphotype in aquatic microbial food webs and to study carbon flow through different bacterial taxa in more detail.

manuscript V: Niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake (Salcher et al. submitted to *Environ Microbiol)*

In this submitted manuscript we investigated the diversity and seasonal as well as vertical population changes of *Betaproteobacteria* in Piburger See. A special focus was put on differences between betaproteobacterial fractions with visible amino acid incorporation. Three main lineages were identified to contribute most to total betaproteobacterial numbers, i.e. beta I (R-BT subclade), beta II (subclades PnecB and PnecC), and beta IV. Numbers of R-BT and PnecB bacteria were rather constant throughout the water column, whereas bacteria affiliated to PnecC and beta IV were particularly numerous in the oxygen-depleted zone of the lake. In general, only moderate seasonal changes were observed in the upper water layers, whereas there was a clear relationship between decreasing oxygen levels and maxima of PnecC and beta IV clades in hypolimnetic water strata. Over 90% of the observed variations in betaproteobacterial numbers and amino acid uptake activity could be attributed to changing oxygen concentrations and the amount of dissolved phosphorus. Almost 80% of R-BT bacteria but less than 15% of cells from the beta IV clade showed amino acid incorporation. Our results suggest that the different betaproteobacterial populations occupy distinct vertical and ecophysiological niches in Piburger See.

manuscript VI: Abundance, biomass, or activity – which parameter mirrors best the standing stock of freshwater bacterial phylotypes? (Salcher et al. in prep)

This study regards the three freshwater bacterial clades *Actinobacteria*, *Betaproteobacteria*, and *Cytophaga-Flavobacteria* and their changing dominances in terms of cell numbers, biomass, and the ability to incorporate amino acids. We showed that the numerical importance of a bacterial lineage need not *per se* mirror its contribution to total bacterial biomass or activity. While *Actinobacteria* formed very high abundances, their contribution to total bacterial biomass was negligible due to their small morphology. In contrast, rare *Cytophaga-Flavobacteria* formed filamentous morphotypes and dominated the bacterial biomass in hypolimnetic, oxygen depleted water. *Betaproteobacteria* contributed most to biomass in the epilimnion because of their relatively high biovolume. Cells with visible amino acid uptake were mainly affiliated with *Actinobacteria* and *Betaproteobacteria*, while

Cytophaga-Flavobacteria showed virtually no incorporation of the offered substrates. Sequence analysis of four PCR based 16S rRNA clone libraries resulted in high numbers of clones affiliated to *Cytophaga-Flavobacteria* undetected by the general probe CF319a. Therefore, the contribution of *Cytophaga-Flavobacteria* or other orders (e.g. *Sphingobacteriales*) within *Bacteroidetes* to total cell numbers and biomass seemed to be underestimated. We stressed the need to analyze not only the abundances of freshwater bacteria, but also their biomass and their incorporation abilities of various substrates to get a comprehensive picture of their ecological importance.

3.2.1. Unpublished data

We studied the seasonal development of bacterioplankton in Piburger See in relation to biotic (i.e. phytoplankton and heterotrophic nanoflagellates) and abiotic (i.e. physio-chemical parameters) factors for a period of one year (February 2005-February 2006). Four depths (3, 9, 18, and 24 m depth) were analysed at monthly sampling intervals for the abundance, biomass, and incorporation of radiolabeled amino acids (as determined by microautoradiography coupled with CARD-FISH) of bacteria from the phyla *Actinobacteria*, *Betaproteobacteria*, and *Cytophaga-Flavobacteria*.

Seasonality of bacteria in relation to biotic and abiotic factors.

Piburger See was covered with ice from the beginning of our seasonal survey in February 2005 until March 2005 and again from December 2005 until the end of the investigation period in February 2006. Spring mixis occurred in April 2005, however, the oxygen profile revealed just a partial oxygen refreshment of the hypolimnion, i.e. a partial mixis. The lake showed a stable thermal stratification during May-November 2005, where the metalimnion reached from 3 m depth in May to 12 m in November. Oxygen was strongly depleted in the hypolimnion during this stratification period, with an anoxic water body reaching from 24 m up to 18 m in November (Fig. 4). Autumnal mixis occurred in early December; however, it was not possible to take samples during this period due to instable ice coverage. Samples of January 2006 showed oxygen refreshment down to at least 21 m depth, which is typical for the period after holomixis. Phosphorus concentrations ranged from 1.7 µg dissolved P l^{-1} in 3 m depth in August to a maximum of 40.9 µg P l^{-1} in 24 m depth in March (Fig. 4). Nitrate was consumed presumably by algae in the upper layers of the lake. Concentrations of nitrate strongly decreased in the hypolimnion in parallel with the progressing oxygen depletion, and an increase of ammonium was found during late summer and autumn (Fig. 7). The low redox potential led to decreasing concentrations of sulphate in 24 m depth in autumn (annual mean: 6.3 mg l^{-1}; minimum: 1.5 mg l^{-1} in 24 m depth in November; data not shown). Dissolved organic carbon accumulated in epilimnion in summer and autumn. Low concentrations (>2 mg l^{-1}) were found in the deeper water column, except for samples close to the sediment (max. 2.4 mg l^{-1}).

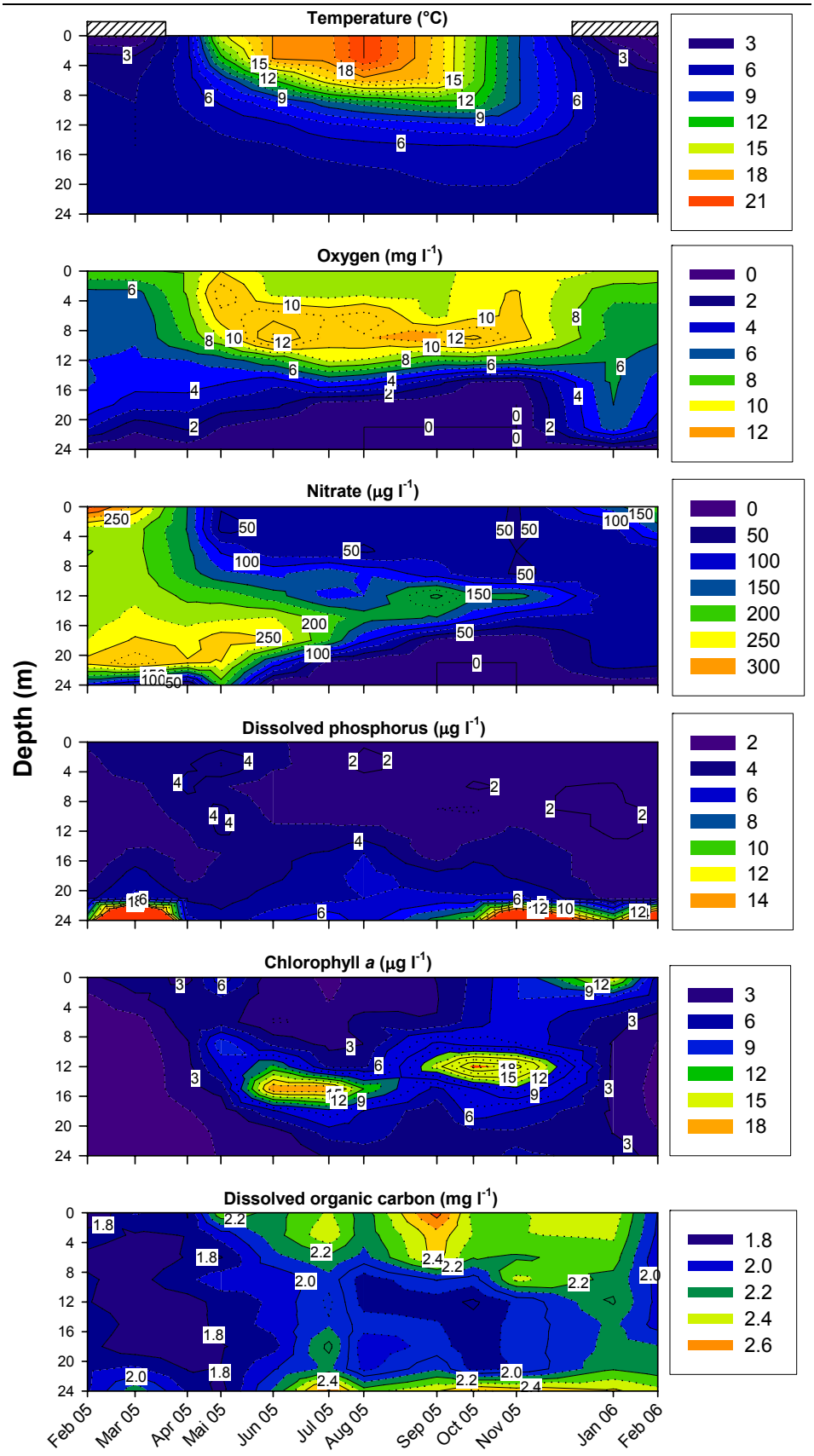

Figure 4: Physical and chemical parameters in Piburger See during the seasonal study in 2005 (modified from Salcher et al. submitted - paper V).

Several pronounced phytoplankton blooms were observed during 2005 (Figs. 4 & 5). The first maximum of algal biomass developed under ice in March (mainly consisting of *Cryptomonads*). The spring bloom in a depth of 9-18 m in April was also dominated by *Cryptomonads*. Two pronounced peaks of chlorophyll *a* concentrations followed in 15 m depth in June-July and in 12-15 m depth in September-November, respectively (Figs. 4 and 5). The first maximum could be attributed to *Chrysophyceae*, whereas the latter almost exclusively consisted of the diatom *Asterionella* sp..

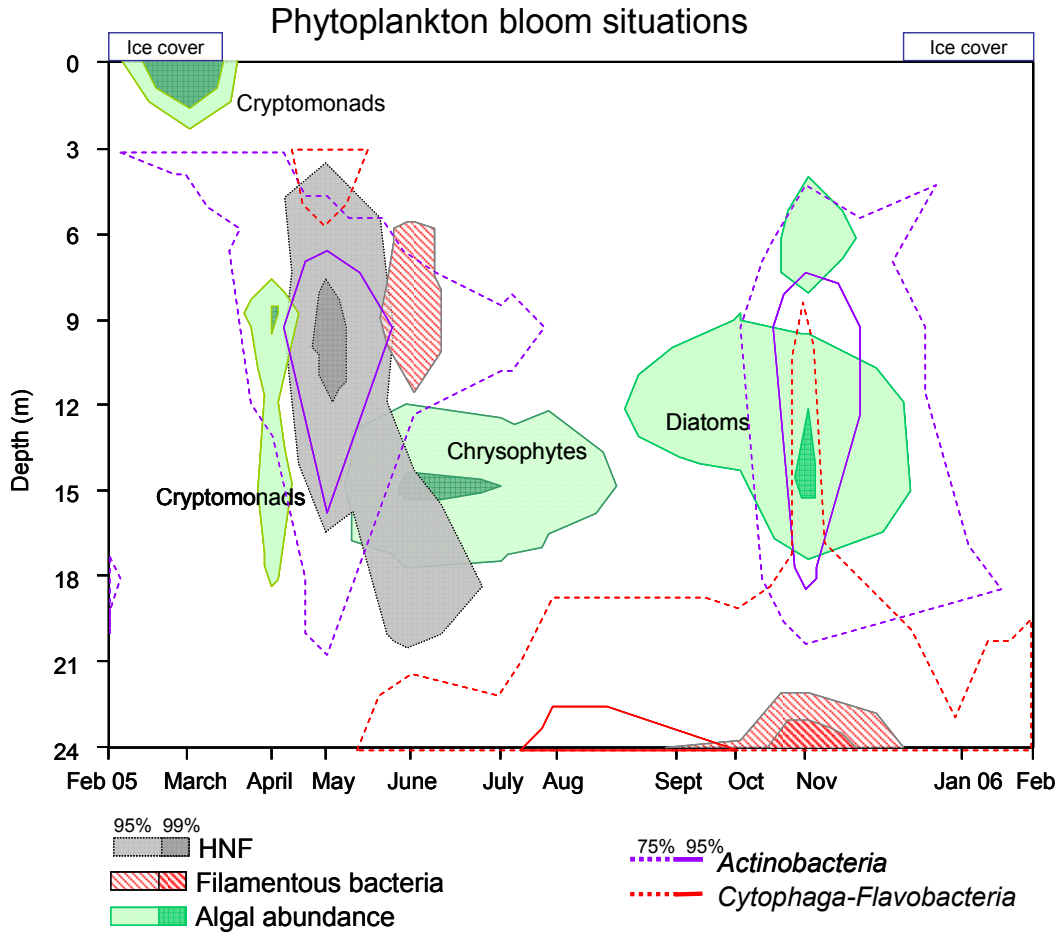

Figure 5: Percentile distribution (95 and 99% of HNF, bacterial filaments, and algal abundances; and 75 and 95% of relative abundances of *Actinobacteria* and *Cytophaga-Flavobacteria* (% of EUB)) during the investigation period in Piburger See.

After the phytoplankton spring bloom, a peak of heterotrophic nanoflagellates (HNF) was detected in 9 m depth, which was followed by a pronounced maximum of filamentous bacteria one month later (Fig. 5). These filaments could not be attributed to any of the studied groups and also not to *Alpha*- or *Gammaproteobacteria* (FISH probes ALF968 and GAM42a, respectively). Filamentous morphotypes of a second

maximum in October-November in 24 m depth were mainly affiliated with *Cytophaga-Flavobacteria*.

Actinobacteria reached highest abundances concomitantly with the HNF peak in May and the bloom of *Asterionella* sp. in November (Figs. 5 & 6 upper panel). In contrast, *Betaproteobacteria* decreased in numbers and amino acid uptake during the period of intense protistan bacterivory (Fig. 6 upper and middle panel), while the fraction of active *Actinobacteria* increased (Fig. 6 middle panel). At the same time, bacterial bulk amino acid uptake and amino acid uptake per cell (per DAPI-stained bacterium with visible incorporation) decreased drastically after a short maximum in April (Fig. 6 lower panel).

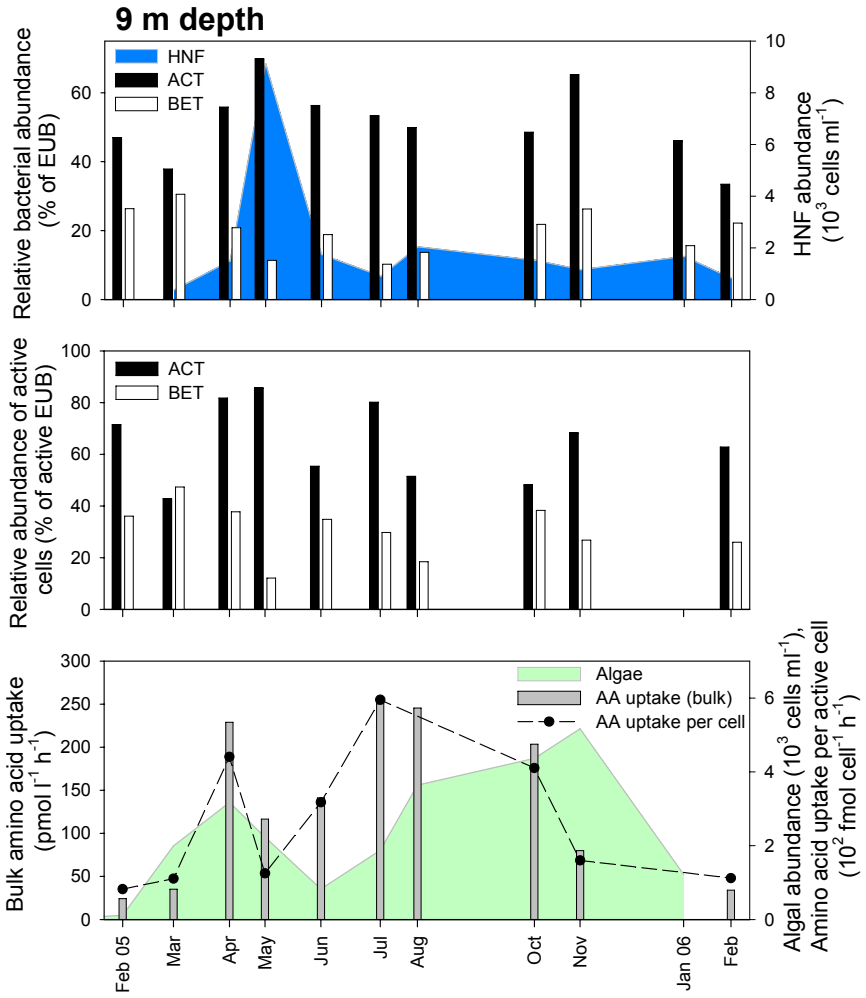

Figure 6: Seasonal changes of bacteria, protists, and algae in 9 m depth.

Upper panel: Relative abundances of Actinobacteria (ACT) and Betaproteobacteria (BET), and flagellate numbers (HNF). Middle panel: Relative abundance of cells with visible amino acid incorporation (in % of EUB active) of ACT and BET. Lower panel: Bacterial bulk amino acid incorporation (AA uptake bulk), amino acid uptake per active cell (AA uptake per cell), and algal abundances.

Spatial distribution of bacteria from different clades in relation to oxygen amounts.

Pronounced physico-chemical depth gradients developed during summer stratification (May-November), with a micro- to anaerobe water body rich in dissolved phosphorus and ammonium (Figs. 4 & 7). This depth gradient was also mirrored in the distribution patterns of bacteria affiliated to the studied lineages (Figs. 7-10). While *Actinobacteria* dominated the assemblage in oxygen-rich water layers, *Betaproteobacteria* formed highest abundances in 24 m depth. The contribution of *Cytophaga-Flavobacteria* to bacterial community was always low, although they were more abundant in oxygen-depleted water (Figs. 7 & 8). A clear rise in total cell numbers occurred concomitantly with a decline of oxygen concentrations in 18 m depth (Fig. 8). Bacterial biomass was highest in hypolimnetic water, where *Cytophaga-Flavobacteria* dominated the bacterial carbon pool (Figs. 7 & 9). These bacteria formed filamentous morphotypes in anoxic samples which were rare (11% of all *Cytophaga-Flavobacteria*) but had extraordinary high cell volumes. *Betaproteobacteria* also contributed more to bacterial biomass in the hypolimnion than in the epilimnion. Although *Actinobacteria* numerically dominated, they typically formed very small morphologies, i.e. minute cocci and vibrios. Therefore, their contribution to total bacterial biomass was always very low. Most bacteria with visible amino acid incorporation were *Actinobacteria* in the epilimnion and *Betaproteobacteria* in the hypolimnion, whereas almost no *Cytophaga-Flavobacteria* incorporated this substrate. Again, a strong increase of all active cells was obvious when oxygen concentrations decreased (Fig. 10).

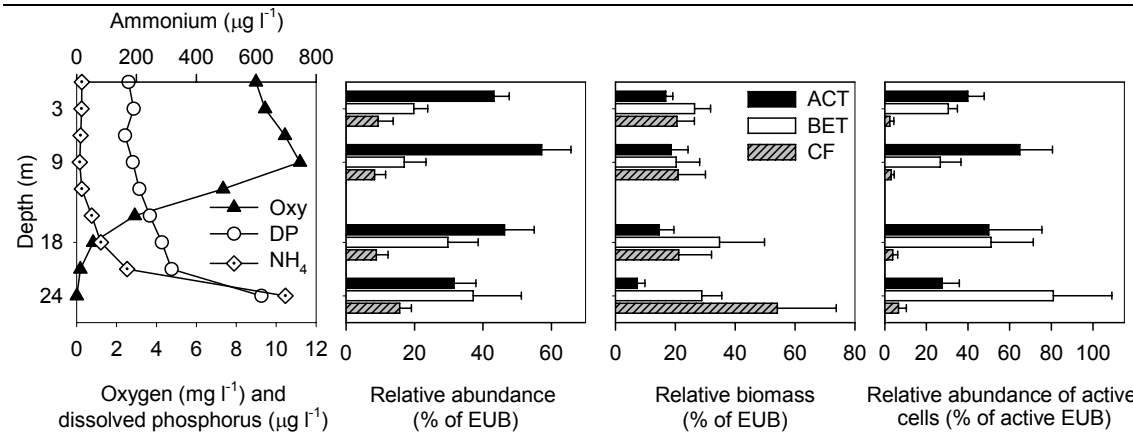

Figure 7: Depth gradients of oxygen, dissolved phosphorus (DP), and ammonium (NH_4) concentrations, and relative abundances, biomasses, and numbers of bacteria incorporating amino acids of *Actinobacteria* (ACT), *Betaproteobacteria* (BET), and *Cytophaga-Flavobacteria* (CF) during summer stratification (May-November 2005). Values are means (n=6), standard deviations are given for the bacterial groups.

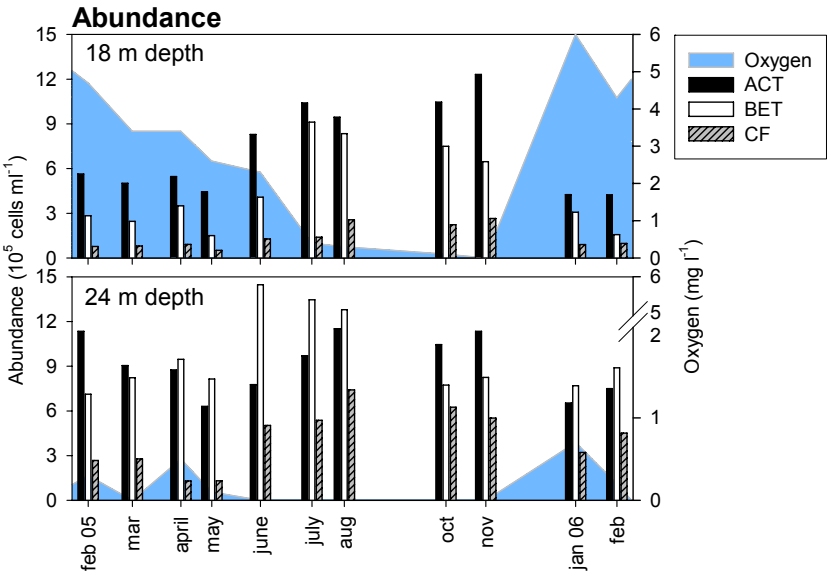

Figure 8: Seasonal changes of bacterial abundances of *Actinobacteria* (ACT), *Betaproteobacteria* (BET), and *Cytophaga-Flavobacteria* (CF) in 18 and 24 m depth in relation to oxygen concentrations ($mg\ l^{-1}$).

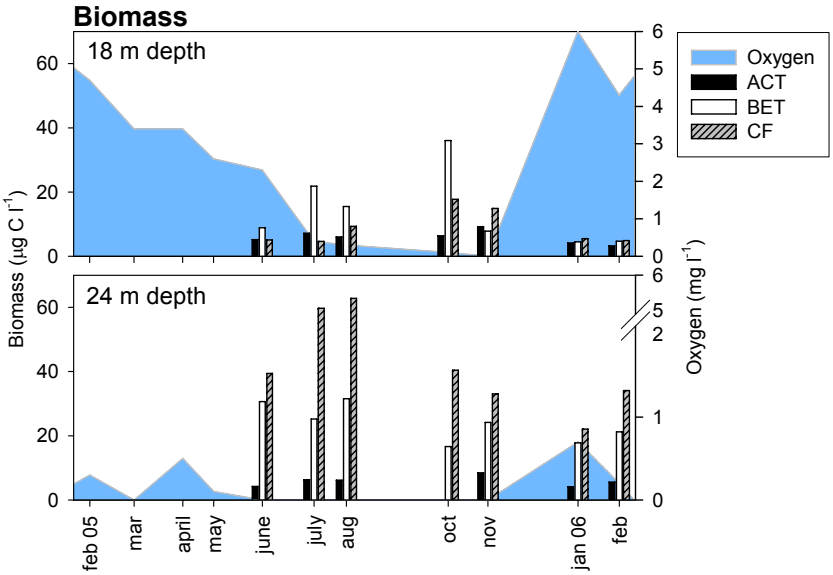

Figure 9: Seasonal changes of bacterial biomass of *Actinobacteria* (ACT), *Betaproteobacteria* (BET), and *Cytophaga-Flavobacteria* (CF) in 18 and 24 m depth in relation to oxygen concentrations ($mg\ l^{-1}$). No biomass evaluation was carried out for samples from February to May 2005.

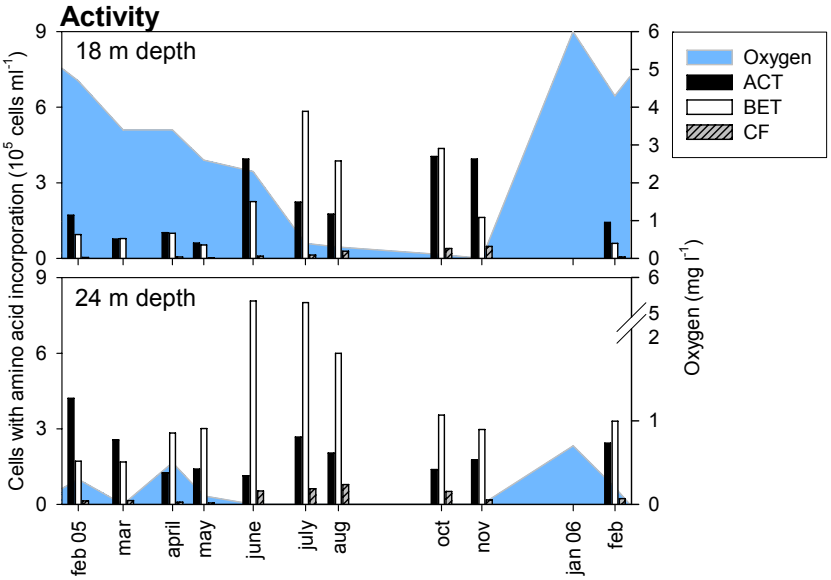

Figure 10: Seasonal changes of cells with visible amino acid incorporation within *Actinobacteria* (ACT), *Betaproteobacteria* (BET), and *Cytophaga-Flavobacteria* (CF) in 18 and 24 m depth in relation to oxygen concentrations ($mg\ l^{-1}$). Microautoradiography was not done in January 2006.

	Oxygen	NH_4	DP
Abundances (cells ml^{-1})			
EUB	**-0.720**	**0.593**	**0.713**
ACT	**-0.527**	**-**	**0.555**
BET	**-0.855**	**0.592**	**0.669**
CF	**-0.741**	**0.715**	**0.721**
Biomass (µg C l^{-1})			
EUB	**-0.828**	**0.618**	**0.688**
ACT	-	-	-
BET	**-0.869**	**0.658**	**0.744**
CF	**-0.830**	**0.808**	**0.771**
Active cells (cells ml^{-1})			
EUB	**-0.575**	**0.592**	0.480
ACT	-	-	-
BET	**-0.715**	**0.579**	0.483
CF	**-0.598**	**0.609**	0.474

Table 2: Pearson's correlation coefficients for abundances, biomasses, and numbers of active cells of all *Bacteria* (EUB), *Actinobacteria* (ACT), *Betaproteobacteria* (BET), and *Cytophaga-Flavobacteria* (CF) in comparison to concentrations of oxygen (Oxygen), ammonia (NH_4), and dissolved phosphorus (DP). Data were log(x+1) transformed prior to analysis. Only coefficients with a significance level $p < 0.005$ are shown, bold values indicate $p < 0.001$.

Discussion of unpublished data

Seasonality of bacterioplankton assemblages in the epilimnion of Piburger See

Actinobacteria showed pronounced seasonal fluctuations in the epilimnion with two maxima in spring and autumn (Figs. 5 and 6). These peaks concomitantly occurred at times of intense grazing by heterotrophic nanoflagellates and a bloom of the diatom *Asterionella* sp., respectively. A protection to protistan grazing by *Actinobacteria* was also reported by other authors (Pernthaler et al. 2001, Hahn et al. 2003), mainly due to their minute cell sizes. Burkert and coworkers (2003) found a maximum of *Actinobacteria* in autumn in Lake Grosse Fuchskuhle, whereas Glöckner et al. (2000) reported on maximum numbers in spring in Gossenköllesee. Allgaier & Grossart (2006) observed two peaks in spring and autumn. The autumnal maximum of *Actinobacteria* in Piburger See could be explained by high amounts of dissolved organic material released by *Asterionella* sp. (e.g. low molecular weight compounds such as sugars), favouring primarily actinobacterial growth. Moreover, *Asterionella* sp. can grow at very low phosphorus concentrations (Tilman et al. 1982, Michel et al. 2006), consequently acting as an effective sink for dissolved phosphorus (Ferris & Lehman 2007). At such low phosphorus concentrations *Actinobacteria* could have a competitive advantage over *Betaproteobacteria*, which show higher growth rates at high phosphorus concentrations (Šimek et al. 2006, Salcher et al. 2007 – paper II).

The low numbers of *Betaproteobacteria* concomitantly with high abundances of flagellates (Fig. 6) may indicate high vulnerability to protistan predation (Jezbera et

al. 2006). Their high numbers in the hypolimnion (Figs. 7 & 8) seemed to be coupled to decreasing oxygen concentrations, but the high amounts of nutrients, esp. phosphorus could also favour their growth. This 'opportunistic life strategy' seemed to hold true at least for the R-BT subcluster of beta I bacteria (Šimek et al. 2006), and partly also for the beta II clade (Burkert et al. 2003). However, as *Betaproteobacteria* represented a very diverse phylum with at least three ecologically distinct groups present in freshwater (Salcher et al. submitted – manuscript V), no generalized conclusions should be drawn by regarding them as a single unit.

A pronounced maximum of filamentous bacteria was detected in the month following the peak of HNF numbers in May. Filaments represent an efficient protection against size selective protistan grazing (Hahn et al. 1999, Langenheder & Jürgens 2001, Pernthaler et al. 2004, Corno & Jürgens 2006). However, the observed filamentous bacteria could not be identified by any of the applied FISH-probes, as was found for filamentous morphotypes dominating in acidified lakes (Vrba et al. 2003). Sequencing of 16S rRNA clone libraries resulted in very high numbers of clones affiliated with *Bacteroidetes* (see Fig. 3 in Salcher et al. in prep – manuscript VI). However, the majority of them showed one or several mismatches with the general oligonucleotide probe CF319a (Manz et al. 1996). This probe does not cover the entire branch of the phylum *Bacteroidetes.* An online probe match at the Ribosomal Database Project II (http://rdp.cme.msu.edu) revealed that only 38% of all available sequences ≥1200 bases affiliated with *Bacteroidetes* were targeted by probe CF319a, and therein mainly *Sphingobacteria* and *Bacteroidales* were missing, whereas 92% of all *Flavobacteria* could be detected theoretically. However, the application of the seemingly more suitable probe CFB560 (O'Sullivan et al. 2002) resulted in no hybridization signals at all (data not shown). Sequences with mismatches to probe CF319a were also not affiliated with the filamentous SOL cluster (*Saprospiraceae, Sphingobacteria;* Schauer et al. 2006) or *Alphaproteobacteria*. Therefore, the affiliation of these filamentous bacteria remains to be elucidated, although there are strong indications for an undetected cluster within *Bacteroidetes*. Thus, abundances and biomasses of *Cytophaga-Flavobacteria* in Piburger See might be even higher than observed.

Spatial distribution of bacterioplankton assemblages

Oxygen, but also nutrients concentrations (i.e. ammonium and dissolved phosphorus) were the most important factors affecting the establishment of bacterial abundances, biomasses, and the numbers of active cells of the studied phylogenetic clades (Table 2). Higher abundances and biomasses of microorganisms in deeper water layers have been reported by Cole et al. (1993). Moreover, specialized microbes (i.e. anaerobes, fermenting or methylotrophic bacteria) would be expected in this anoxic ecosystem (Casamayor et al. 2000; Lehours et al. 2007).

Except for *Actinobacteria*, all lineages were present in higher numbers, biomasses, and active cells in the oxygen depleted hypolimnion. Therefore, it is likely that some *Betaproteobacteria* and *Cytophaga-Flavobacteria* are capable of an obligate or facultative anaerobic metabolism (Alonso & Pernthaler 2005). Two clusters of *Betaproteobacteria* contributed the largest part to the bacterial maximum in the oxygen depleted hypolimnion of Piburger See (i.e. beta IV and PnecC bacteria, see Salcher et al. submitted – manuscript V). *Cytophaga-Flavobacteria* in this anoxic environment may also represent different species than in the epilimnion. However, sequence analysis of 16S rRNA clone libraries revealed no genotypes specific for anoxic samples (Salcher et al. in prep – manuscript VI). High abundances of *Cytophaga-Flavobacteria* in anoxic layers of lakes were also reported by Glöckner et al. (1999) and Casamayor et al. (2000). The high biomass of *Cytophaga-Flavobacteria* in anoxic samples was due to the formation of filaments by these bacteria (Figs. 7 & 9). So far, filamentous morphotypes in freshwater habitats were mainly found to be affiliated with *Cytophaga-Flavobacteria* and *Sphingobacteria* (Alfreider et al. 1996, Pernthaler et al. 1998, Pernthaler et al. 2004, Schauer & Hahn 2005, Šimek et al. 2007) but *Alpha*- and *Betaproteobacteria* may also form filaments (Salcher et al. 2007 – paper II, Nishimura & Nagata 2007). One study even reports on filamentous *Actinobacteria* in a drinking water reservoir (Nielsen et al. 2006). In our study, filamentous *Betaproteobacteria* were rarely detected in the hypolimnion, whereas a large fraction of filaments could be attributed to *Cytophaga-Flavobacteria*.

A higher activity of *Actinobacteria* than of all *Bacteria* (EUB positive) in the assimilation of thymidine was detected in a drinking water reservoir (Nielsen et al. 2006), whereas similar DNA *de novo* synthesis rates for *Actinobacteria* and all *Bacteria* were reported for several high mountain lakes (Warnecke et al. 2005). *Betaproteobacteria*, and therein especially the R-BT subcluster of beta I showed very

high amino acid incorporations (Horňák et al. 2006, Pérez & Sommaruga 2007, Salcher et al. submitted – manuscript V). The overall low uptake of amino acids by *Cytophaga-Flavobacteria* corresponded to the findings of Cottrell & Kirchman (2000), that marine and estuarine *Cytophaga-Flavobacteria* are underrepresented in the proportion of amino acid incorporating bacteria. Comparable low leucine uptake rates by this phylum were also found for two freshwater ecosystems (Horňák et al. 2006, Pérez & Sommaruga 2006). Marine *Cytophaga-Flavobacteria* seemed to preferably incorporate high-molecular weight compounds such as proteins, cellulose, or chitin (Cottrell & Kirchman 2000), and they played an important role in the degradation of dissolved organic matter and carbon fluxes (Kirchman et al. 2002). Therefore, the low amino acid incorporation of this group may not reflect their activity; the choice of different substrates could have resulted in opposing findings. Moreover, it should be taken in account that not all of the offered substrate may be used for bacterial biomass production, i.e. amino acids could also be respired for energy supply. Around one third of the gross uptake of ^{14}C amino acids was found to be respired by bacteria in Lake Constance with high seasonal variations (Weiss & Simon 1999), and even higher respiration rates of ^{3}H labelled amino acids were detected in the oligotrophic Sargasso Sea (60 - 80%; Suttle et al. 1991) and coastal marine waters (20 - 60%; Carlucci et al. 1984).

Although bacteria of the studied freshwater phyla *Actinobacteria*, *Betaproteobacteria*, and *Cytophaga-Flavobacteria* comprise a high diversity, it seems that they differ profoundly in general properties such as cell size or the incorporation of amino acids.

Summary of part II

A detailed one year survey at Piburger See revealed pronounced temporal and spatial variations of freshwater bacteria affiliated with *Actinobacteria*, *Betaproteobacteria*, and *Cytophaga-Flavobacteria*. Bacteria of these three lineages were identified as dominant in terms of abundances, biomasses, or amino acid incorporation. Hybridization with the general probe EUB I-III (targeting all *Bacteria*) covers the bulk of total bacterial biomass, even though the numerical detection efficiency was only around 50% of all DAPI-stainable particles. The contribution of the studied phyla to total bacterial biomass did not agree with their numerical importance, e.g. although *Actinobacteria* clearly dominated the bacterial assemblage, their contribution to biomass was negligible due to their generally small size. In contrast, *Cytophaga-Flavobacteria* were present in very low abundances, but contributed disproportionally to bacterial biomass because they formed very large morphotypes such as filaments. Bacteria with amino acid incorporation were mainly affiliated with *Actinobacteria* and *Betaproteobacteria*, whereas *Cytophaga-Flavobacteria* showed virtually no uptake of the offered substrate. The phylogenetic diversity of *Betaproteobacteria* was further studied in detail for their abundances and amino acid incorporation. Three lineages were identified as being numerically important in Piburger See, i.e. the beta I (R-BT sublineage), beta II (PnecB and PnecC sublineages), and beta IV clades. Pronounced differences in amino acid incorporation were observed, where beta I bacteria were highly active (almost 80 % of these bacteria took up amino acids), whereas almost no beta IV bacteria incorporated this substrate. Distinct vertical distribution patterns occurred during summer stratification. In parallel with oxygen depletion of hypolimnetic water strata, a bloom of bacteria affiliated with PnecC (beta II) and beta IV was observed. Therefore, we concluded that bacteria of these betaproteobacterial clades occupy distinct spatial niches within the ecosystem Piburger See.

3.3. General conclusions and discussion

By our experimental approaches we were able to identify bacteria with a high vulnerability to grazing by heterotrophic nanoflagellates and others with high grazing resistance (papers I-III). We demonstrated that the concentrations of limiting nutrients such as phosphorus can lead to pronounced shifts in the composition of the bacterial assemblage due to competitive advantages or disadvantages of microbes from different clades (papers II & III). In order to set our experimental data in a wider context, we studied seasonal and spatial variations of the bacterioplankton assemblage in a detailed one year survey at Piburger See with a special focus on bacterial abundances, biomasses, and the proportion of cells with amino acid incorporation (manuscripts V & VI).

Betaproteobacteria were identified as being highly vulnerable to grazing by heterotrophic protists, but at the same time very active and fast growing when nutrients such as phosphorus were available (papers I-III). Some bacteria within this phylum (esp. the R-BT sublineage of beta I) rapidly dominated the assemblage at surplus phosphorus concentrations, but were limited by top-town control (Jezbera et al. 2006, Šimek et al. 2006, papers II & III). These bacteria therefore may follow the strategy of so-called 'uptake specialists' (Thingstad et al. 2005). This strategy is characterized by fast growing bacteria with small cell sizes, which favour the diffusive nutrient transport. A 'killing the winner' mechanism acting in parallel (Thingstad & Lignell 1997) balances the co-existence with the so-called 'defense specialist', and the biomass of the 'uptake specialist' is regulated by predator or parasite control. In contrast, the resource controlled 'defense specialist' is characterized by a high predation resistance regulated by cell size (Thingstad et al. 2005), i.e. minute or enlarged cell sizes which seem to serve as efficient defense strategies (Hahn & Höfle 2001). *Actinobacteria* might fall into this category, as their small cell size may prevent these microbes from ingestion by flagellates (Pernthaler et al. 2001).

Four phylotypes of *Betaproteobacteria* within three ubiquitous freshwater clades (i.e. the beta I, beta II, and beta IV clade; Glöckner et al. 2000) were identified in Piburger See, occupying distinct temporal and spatial niches, which were particularly separated by declining oxygen concentrations in the hypolimnion (manuscript V). Bacteria of the already mentioned R-BT sublineage (beta I) and bacteria of the PnecB sublineage (beta II) were equally distributed over the whole water column, whereas a distinct bloom of microbes affiliated with PnecC and beta IV

was found in anoxic water strata. These bacteria might be able of a facultative or obligate anaerobe lifestyle (Alonso & Pernthaler 2005). Moreover, bacteria from the different betaproteobacterial clades displayed contrasting incorporation of amino acids, including one lineage with very active microbes (i.e. the R-BT sublineage; Horňák et al. 2006; Pérez & Sommaruga 2006) and one clade comprising most likely methylotrophic bacteria (i.e. the beta IV clade, related to *Methylophilus* sp.) which did not incorporate the offered substrate (manuscript V). Therefore, *Betaproteobacteria* seem to be a very diverse phylum which harbours ecophysiologically different freshwater phylotypes.

Actinobacteria, typically known as soil inhabitants (Goodfellow & Williams 1983), are gram positive bacteria with a high genomic G+C content. Members of freshwater *Actinobacteria* are globally distributed (Glöckner et al. 2000, Warnecke et al. 2004), and were found in very high numbers in a wide range of different lakes (Sekar et al. 2003, Warnecke et al. 2005, Allgaier & Grossart 2006). These bacteria belonged to the dominant fraction of the bacterioplankton in high mountain lakes (Warnecke et al. 2005) and also in Piburger See (Sekar et al. 2003, manuscripts IV & VI). Some species within this monophyletic group seemed to be protected by protistan grazing due to their small size and their cell wall structure (Pernthaler et al. 2001, Hahn et al. 2003, Jezbera et al. 2006). *Actinobacteria* showed no response to flagellate grazing and / or surplus amounts of phosphorus in our experimental study in Piburger See (papers II & III). However, they seemed to be more competitive at times of high grazing pressure (after phytoplankton spring bloom) and low nutrient concentrations during an autumnal bloom of the diatom *Asterionella* sp. (unpublished data, Figs. 5 & 6). These bacteria exhibited pronounced seasonal fluctuations in the epilimnion of Piburger See, but were not affected by the changing oxygen regime in the hypolimnion (unpublished data, Fig. 7). Although *Actinobacteria* numerically dominated in Piburger See, their contribution to total bacterial biomass was almost negligible due to their constantly small size (Glöckner et al. 2000, manuscripts IV & VI). *Actinobacteria* represented the highest activity (here defined as amino acid incorporation) to biomass ratios (manuscript VI), thus, they may play a very important role in the remineralization of low molecular weight compounds of the organic carbon pool (Warnecke et al. 2005).

In contrast, *Cytophaga-Flavobacteria* represented an altogether very rare lineage in Piburger See, but with higher abundances in the anoxic hypolimnion,

where they even dominated the assemblage in terms of bacterial biomass (manuscript VI). These bacteria formed very large morphotypes such as filaments (Alfreider et al. 1996, Pernthaler et al. 1998, manuscripts IV & VI), which seemed to convey a resistance against protistan grazing (Šimek et al. 2007, papers I-III). *Cytophaga-Flavobacteria* showed almost no incorporation of radiolabeled amino acids (Horňák et al. 2006, Pérez & Sommaruga 2006, manuscript VI). Marine representatives of this phylum preferably incorporated high molecular weight compounds of the dissolved organic carbon pool (Cottrell & Kirchman 2000). Some marine members of *Cytophaga-Flavobacteria* seem to play an important role in the degradation of complex and polymeric organic matter (Kirchman 2002). This might also be the case for freshwater *Cytophaga-Flavobacteria*, although they phylogenetically differed from their marine counterparts (Alonso et al. 2007). Thus, we recommend studying the incorporation preferences of freshwater *Cytophaga-Flavobacteria* for different components of the organic carbon pool, thus elucidating their ecological function. Moreover, abundances and biomass of *Cytophaga-Flavobacteria* and other members of *Bacteroidetes* seemed to be underestimated in Piburger See, as the general FISH probe CF319a (Manz et al. 1996) covers only a part of all available sequences (i.e. 38%, unpublished data). Sequencing of clone libraries resulted in a very high number of clones affiliated with *Bacteroidetes* that were not detected by this probe (manuscript VI). Therefore, the design and application of FISH probes with a better coverage or the detection of phylotypes not covered by probe CF319a are highly recommended.

Alpha- and *Gammaproteobacteria* were not further analyzed in the seasonal study, as their numbers were almost below detection limit (manuscript VI). However, *Alphaproteobacteria* were able to establish high numbers in the presence of protistan predators and adequate nutrient supply (Jürgens et al. 1999, papers II-III). Bacteria of this phylum can react to grazing by filament or aggregate formation (Jürgens et al. 1999, Langenheder & Jürgens 2001, papers I-III).

To conclude, we were able to identify major bacterial lineages present in Piburger See and their response to top-down and bottom-up factors. We highlight, that these factors have a pronounce impact on the competitive abilities of different bacterial lineages which resulted in changes in the bacterial assemblage. However, more research is needed to gain a comprehensive view on the dynamics of freshwater bacteria from different clades.

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5. Publications

5.1. Contribution to the publications

paper I

Concept by TP & MS, experimental design, counting and biomass evaluation of microorganisms by TP & MS, CARD-FISH and biomass evaluation of hybridized bacteria by MS, evaluation and discussion of data by MS, TP, JP & RP, writing of the manuscript by MS

paper II

Concept by TP, JV & KŠ, experimental design by TP, KŠ, KH & JJ, counting and biomass evaluation of microorganisms by BSo & TP, FLB evaluation by JJ & KŠ, CARD-FISH and biomass evaluation of hybridized bacteria by MS, flow-cytometric measurements by JH, evaluation and discussion of data by MS, JV, KŠ & TP, writing of the manuscript by MS

paper III

Concept by TP, JV & KŠ, experimental design by TP, KŠ, KH & JJ, counting and biomass evaluation of microorganisms by BSo & TP, FLB evaluation by JJ & KŠ, CARD-FISH and biomass evaluation of hybridized bacteria by MS, phosphorus measurements by BM, bacterial bulk production by BSa, evaluation and discussion of data by MS, JV, KŠ & TP, writing of the manuscript by TP

manuscript IV

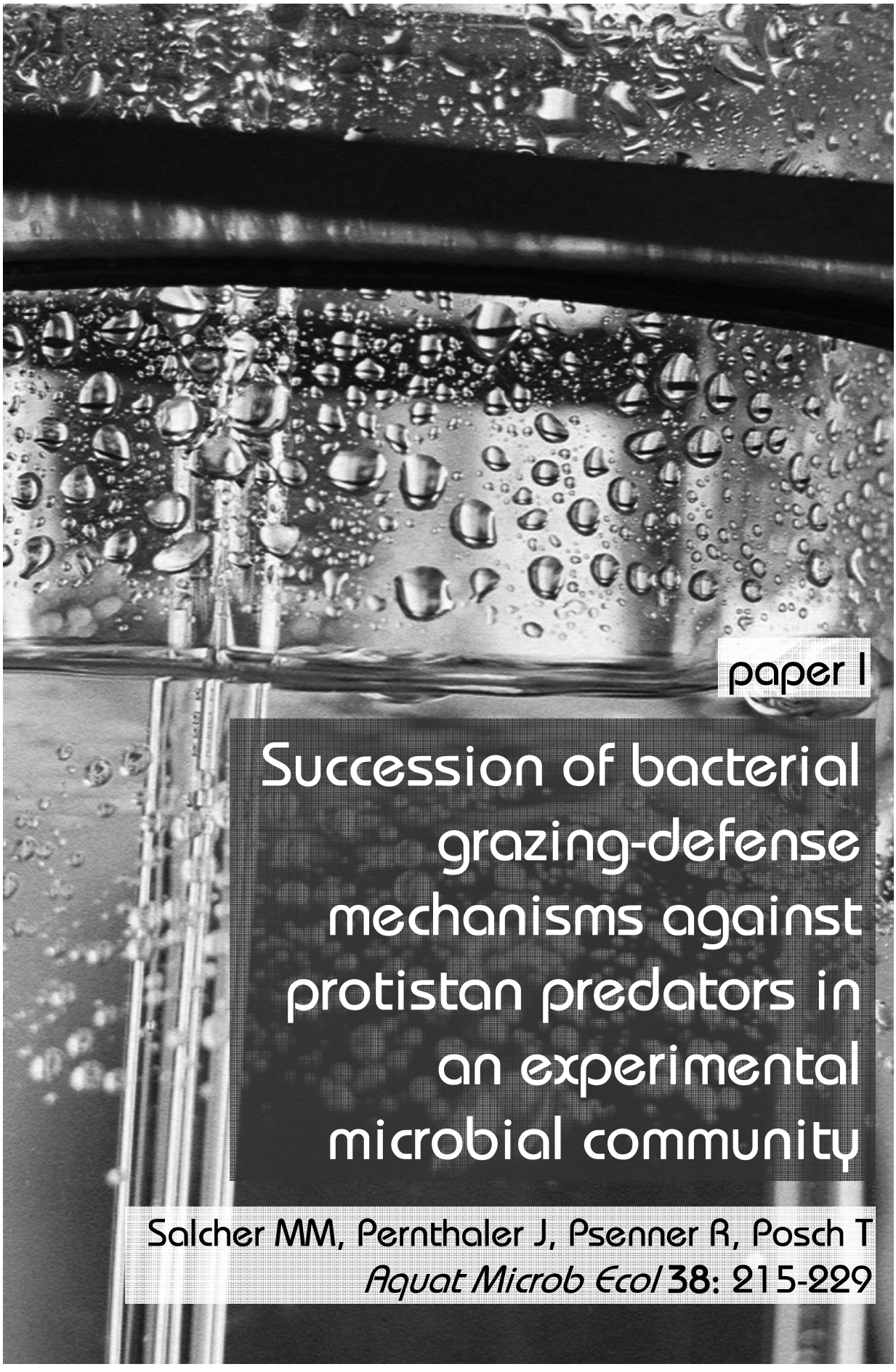
Concept by TP & MS, sampling by JF, MS & TP, counting and biomass evaluation of bacteria by TP, method development by TP, CARD-FISH and biomass evaluation of hybridized bacteria by MP & MS, chemical analyses by JF, evaluation and discussion of data by TP, MP & MS, writing of the manuscript by TP

manuscript V

Concept by TP & MS, sampling, and counting of microorganisms by TP & MS, CARD-FISH by MS, MAR-FISH by MS, cloning and sequencing by MS, development of automated microscopy by MZ, evaluation and discussion of data by TP, RP, JP & MS, writing of the manuscript by MS

manuscript VI

Concept by TP & MS, sampling, counting and biomass evaluation of microorganisms by TP & MS, CARD-FISH and biomass evaluation of hybridized bacteria by MS, MAR-FISH by MS, cloning and sequencing by MS, evaluation and discussion of data by TP, JP & MS, writing of the manuscript by MS



paper I

Succession of bacterial grazing-defense mechanisms against protistan predators in an experimental microbial community

Salcher MM, Pernthaler J, Psenner R, Posch T

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Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community

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ABSTRACT: We studied the effects of 2 bacterivorous protistan predators on the phenotypic and taxonomic successions of an experimental bacterial community growing in continuous culture with the cryptophyte *Cryptomonas phaseolus*. Two predators were inoculated to the cultivation system, the mixotrophic flagellate *Ochromonas* sp., an interception feeder, and the filter-feeding heterotrophic ciliate *Cyclidium glaucoma*. The system was sampled every second day over a period of 3 mo. The competition between algae and bacteria for limiting phosphorus (20 µg P l^{-1} d^{-1}) was reflected in pronounced fluctuations of the ratio of algal versus bacterial biomass. High grazing pressure exerted on bacteria by the flagellate led to a strong decrease of bacterial abundance and biomass, with only few cells left freely dispersed. A succession of various grazing-resistant morphologies was observed, such as microcolonies, large aggregates and filaments. A population affiliated with *Aquabacterium* sp. formed grazing-resistant co-colonies with a *Caulobacter* spp., indicating that these 2 bacteria might find a common refuge from protistan grazing within aggregates or biofilms. During the course of the experiment these large colonies were succeeded by grazing-protected morphotypes related to the *Flexibacter sancti–Flavobacterium ferrugineum* lineage of the Sphingobacteriales. In contrast, the ciliate induced only a slight reduction of bacterial abundance, and bacteria even recovered to higher than original numbers after a few weeks. However, pronounced successions of microbial populations were also observed in the presence of the ciliate.

KEY WORDS: Bacteria–algae interaction · Bacterial assemblage composition · Bacterial succession · Continuous cultivation system · *Cyclidium glaucoma* · Grazing defense mechanism · *Ochromonas* sp. · Predator–prey interaction

INTRODUCTION

Grazing by bacterivorous protists can affect major phenotypic and physiological characteristics and the species composition of a bacterial assemblage. Changes in bacterial morphology due to protistan grazing are well documented (for review see Hahn & Höfle 2001, Jürgens & Matz 2002). In contrast, fewer studies have investigated the relationship between genotypic and phenotypic assemblage changes, and different bacteria seem to follow contrasting strategies to respond to a particular predator. Some bacterial phylotypes show pronounced phenotypic plasticity, as reflected by an increase or decrease in size, the formation of filaments or the establishment of microcolonies or aggregates (Hahn & Höfle 2001, Hahn et al. 2004).

Fluorescence *in situ* hybridization (FISH) is a powerful tool to simultaneously study the phenotypic characteristics and phylogenetic identity of cells in mixed bacterial assemblages. Using FISH, grazing-induced changes in bacterial assemblage composition have been repeatedly observed both in the laboratory (Pernthaler et al. 1997, 2001, Šimek et al. 1997, Posch et al. 2001) and in field studies (Jürgens et al. 1999, Šimek et al. 1999, 2001a,b, 2003, Langenheder & Jürgens 2001, Gasol et al. 2002). Such shifts could be caused directly

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by size-selective feeding, but also indirectly, for instance as a result of a change in the abundance of bacterial competitors or in the nutrient release by predators (see Fig. 5 in Jürgens & Matz 2002).

In most of the aforementioned studies, changes in bacterial assemblages caused by protistan grazing have been induced experimentally and they were studied over very short time periods (hours and days). In contrast, our knowledge about successions of distinct bacterial genotypic populations or of their grazing defense mechanisms in response to continuous protistan grazing pressure is still scarce (Masín et al. 2003). There are first indications that grazing-induced shifts of bacterial assemblages are not only linked to the differential vulnerability of individual bacterial populations, but at the same time to the dominance of different predator species (Posch et al. 1999, Pernthaler et al. 2001). Likely, grazing by bacterivorous protists does not provoke a simple binary (yes/no) answer. Instead, the specific characteristics of the grazing regime (e.g. different nutrition modes, different size selectivity, varying generation times of predators, assemblage composition of predators) will be of relevance for the development of any diverse bacterial assemblage. Individual bacterial genotypes might thus be characterized by different levels of vulnerability to grazing per se, but at the same time the grazing-induced responses might be strongly influenced by the characteristics of protistan feeding.

We studied the long-term interactions between a mixed experimental bacterial assemblage and 2 protistan predators with different feeding behavior. Exudates of the autotrophic alga *Cryptomonas phaseolus* were used as a natural carbon source by the accompanying bacteria. The sampling interval was adapted to match the calculated doubling time of organisms as determined by the dilution rate (theoretically 40 bacterial generations).

Our hypotheses were (1) that grazing by 2 protistan species with different feeding modes will cause different responses of the bacterial community, e.g. in abundance, size, biovolume and bacterial assemblage composition; (2) that there will be successions of different bacterial genotypic populations; and (3) that the bacterial assemblage will show a succession of grazing protection mechanisms when exposed to predation over an extended period of time.

MATERIALS AND METHODS

Continuous cultivation system. The experiment was performed in a 2-stage continuous cultivation system. Details of the setup have been described earlier in Posch et al. (1999). Briefly, the first stage (2.4 l, further described as I-Stage) was inoculated with the cryptophyte *Cryptomonas phaseolus* and its accompanying mixed bacterial assemblage (see Pernthaler et al. 2001 for a phylogenetic analysis of dominant community members). The algae were grown on WC-medium (Guillard & Lorenzen 1972) with a decreased phosphorus content of 50 µg P l^{-1} at 18°C in a light:dark rhythm of 16:8 h. Initially, the system was tested with distilled water and autoclaved before inoculation of the *C. phaseolus* and bacteria stockcultures. It was maintained as batch culture for 3 d, and afterwards as a chemostat for 79 d with a dilution rate of 0.39 d^{-1} (corresponding to a P-loading of 19.5 µg P l^{-1} d^{-1}). Three replicate vessels (780 ml each) were used as second stages. Two predator species were inoculated in the second stage vessels at Day 7, the mixotrophic flagellate *Ochromonas* sp. strain DS, an interception feeder (further described as II-Och), and the heterotrophic scuticociliate *Cyclidium glaucoma*, a filter feeder (further described as II-Cyc). The third vessel served as control without any predators (further described as II-Con). The dilution rate of the second stage vessels was 0.35 d^{-1}. Protists were precleaned from bacteria by washing and filtering the stockcultures with sterilized WC-medium onto 3 µm filters and inoculating them in *C. phaseolus* stockculture to remove their accompanying bacteria. This process was repeated 3 times within a month before the experiment was started.

Samples were taken every second day over a period of 79 d in order to follow the theoretical generation time of organisms, given by the dilution rate of the second stage. Sampling was carried out with a special construction to avoid contamination by other bacteria, which is particularly important for long-term experiments. Only a small volume of each vessel was removed every second day (75 ml, <10% of the total vessel volume), so that there was minimal disturbance of the chemostat system by sampling. Counting and sizing of organisms was done within 1 d after sampling.

Determination of bacterial and protistan abundance and biomass. Subsamples of 40 ml were fixed with alkaline Lugol's solution (0.5% final concentration), followed by formaldehyde (3% final concentration), and decolorized with several drops of sodium thiosulfate (Sherr & Sherr 1993). Samples were stained with DAPI (4′,6′-diamidino-2′-phenylindole, 6.7 µg ml^{-1} final concentration) and filtered onto black polycarbonate filters (Osmonics, 0.22 µm pore size, 25 mm diameter). Microscopic evaluation was carried out with a Zeiss Axiophot and a Zeiss Axioplan epifluorescence microscope at a magnification of 1600× and 320× for counting at least 1000 bacteria and 400 protists per sample, respectively. Ten to 20 images of single bacteria (corresponding to 500–700 cells), 50

images of microcolonies in II-Och, and 10 images of *Cryptomonas phaseolus*, *Ochromonas* sp. and *Cyclidium glaucoma* were recorded with CCD cameras (Vosskühler VDS CCD–1300 and Cohu) mounted on the microscopes, and processed with the image analysis program LUCIA_D (http://www.lim.cz). At least 500 to 700 bacteria, 300 algae and 50 predators were measured. Area and perimeter of each cell were measured, and cell length and width and the mean cell volume (MCV) were calculated applying geometric approximation (Massana et al. 1997). Details on image processing and the use of macros can be found in Posch et al. (1997). Bacterial carbon content was calculated using the volume to carbon conversion factor published by Loferer-Krößbacher et al. (1998). Bacterial carbon was estimated to amount for 50% of bacterial dry weight. For the determination of protistan carbon, we applied the formula published by Menden-Deuer & Lessard (2000). Total biomass was acquired by multiplying mean cellular carbon content with total abundance. Bacterial phosphorus content was calculated applying a conversion factor of 2 fg P cell^{-1}, while algal phosphorus content was assumed to be 900 fg P cell^{-1}. These conversion factors have been determined for the same microbial community in earlier experiments (Mindl et al. 2005, this issue). For *Ochromonas* sp., a conversion factor of 303 fg P cell^{-1} was used (Rothhaupt 1996a), whereas no P conversion factor was available for *C. glaucoma*. Therefore, we calculated the ciliate's phosphorus content indirectly via subtraction of bacterial and algal P from the original phosphorus content of 50 µg l^{-1} of the medium. Running averages were calculated by averaging 3 consecutive sampling dates (6 d).

Counting and sizing was done in triplicates at 2 sampling occasions, with 3 parallel sampling bottles and 3 parallel preparations for all bacterial and protistan parameters. Statistical comparisons of parameters of cell morphology and biomass were performed by 1-way ANOVA.

Determination of bacterial assemblage composition. Five to 7 ml of the fixed subsample (see above) were filtered onto white polycarbonate filters (Millipore, Type GTTP, 47 mm diameter, 0.2 µm pore size), rinsed with distilled water, and stored at –20°C until further processing. Sampling dates were selected for hybridization if significant changes were found either in bacterial or protistan abundance of II-Och or II-Cyc (altogether 7 timepoints).

Fluorescence *in situ* hybridization (FISH) was either carried out with fluorescently monolabeled oligonucleotide probes or with horseradish peroxidase labeled probes and catalyzed reporter deposition (CARD) (Pernthaler et al. 2002). No differences between detection rates by the 2 methods were found in the experimental assemblage (Sekar et al. 2003). CARD–FISH was performed with the probe HGC-69a, targeted to the *Actinobacteria* (Sekar et al. 2003), with probes ALF-968 and BET-42a, specific for the α- and β-subclasses of Proteobacteria, respectively, and with probe EUB I-III, which detects most bacteria including *Verrucomicrobia* and *Planctomycetes* (Daims et al. 1999, Glöckner et al. 1999). Cell attachment and permeabilization on membrane filters and CARD–FISH was carried out as previously described with fluorescein-labeled tyramides for signal amplification (Pernthaler et al. 2002, Sekar et al. 2003). In addition, 3 specific probes labeled with the fluorescent dye Cy3 were applied for 3 microbial groups previously found in the experimental assemblage (Pernthaler et al. 2001). Probe CAU663 is targeted to bacteria affiliated with the *Caulobacter* spp.–*Brevundimonas* spp. lineage (α-Proteobacteria) (probe sequence: 5'-GGA GTT CCA CAT ACC TCT-3') (Neef 1997). Probe BET3-446 is targeted to an '*Aquabacterium* sp.' (α-Proteobacteria), and CF1-853, specific for bacteria from the *Flavobacterium ferrugineum–Flexibacter sancti* clade of the *Sphingobacteriales* (Pernthaler et al. 2001). FISH of filter sections was carried out as previously described (Alfreider et al. 1996, Glöckner et al. 1996), with the difference that cells had been pretreated as required for the CARD–FISH procedure (Sekar et al. 2003). The formamide concentrations in the hybridization buffers were 30% for probe CAU663, 40% for probe BET3-446, and 35% for probe CF1-853.

The filter sections were counter-stained with DAPI (1 µg ml^{-1}) and inspected with a Zeiss Axiophot epifluorescence microscope at a magnification of 1250×. At least 1000 DAPI-stained cells were counted per sample (500 cells for β-Proteobacteria, which were more frequent), corresponding to 15 to 500 hybridized cells. In the vessel II-Och, where besides single cells, microcolonies and aggregates also appeared, only single cells were analyzed by FISH. In addition, the food vacuoles of *Cyclidium glaucoma* and *Ochromonas* sp. were inspected for hybridized bacteria. For the mixotrophic flagellate this was only feasible using CARD–FISH because the chlorophyll autofluorescence masked the signals of the Cy3 monolabeled probes. To verify the precision of abundance determinations, 3 replicate hybridizations were made from different sampling dates with probes BET3-446 and BET-42a. Ten images of bacteria were recorded with a CCD camera from each filter section. Composite pictures of cells from these images were arranged with the image analysis program LUCIA_D to visualize the succession of morphotypes of different bacterial clades with and without predators and the cell lengths of hybridized cells was measured.

RESULTS

Bacterial and protistan abundance (Fig. 1)

In I-Stage and II-Con, similar patterns occurred in the course of the experiment. Initially the abundance of *Cryptomonas phaseolus* decreased slightly, remained stable until Day 35, and dropped to the lowest value at Day 51. Thereafter, algae recovered, with fluctuating abundances until the end of the experiment. Bacterial abundance showed the opposite trend, a slight initial increase to more or less stable cell numbers in the beginning, followed by a strong rise to a maximum at Day 51. Subsequently, bacterial abundance dropped, remained stable for some days and increased again at the end of the experiment. The ratio of bacterial versus algal biomass changed rapidly between Days 40 and 60, from 0.2 to 0.8, and fluctuated between <0.2 and 0.4 thereafter (Fig. 2).

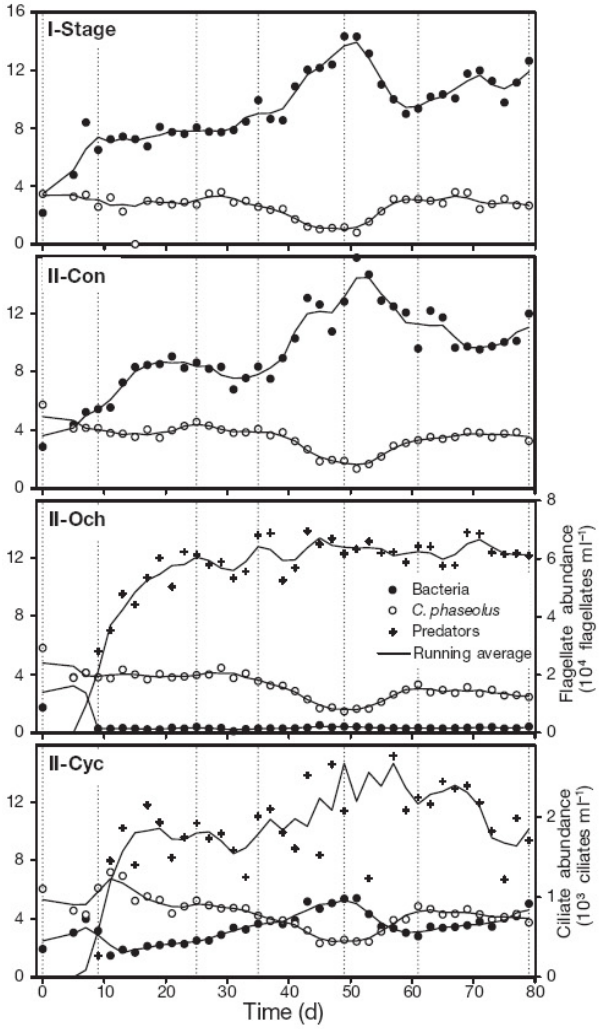

Fig. 1. Bacterial, algal and protistan abundances in the different vessels of the continuous cultivation system. Dotted lines indicate sampling dates selected for fluorescence *in situ* hybridization (FISH) analysis. Note the different units for bacterial and algal abundance on the left *y*-axis. Abbreviations as in Fig. 1 legend

In II-Och, algae followed the same patterns as observed in I-Stage and II-Con. *Ochromonas* sp. grew exponentially until Day 19 and its numbers remained more or less stable thereafter (Fig. 1). Bacterial abundances decreased by more than 1 order of magnitude shortly after inoculation of the predator and remained at this low level (about 8% of the initial abundance) until the end of the experiment.

Algal numbers in II-Cyc showed the same trend as in the other variants, but formed higher abundances than in all other vessels and a short, but pronounced initial peak (Fig. 1). *Cyclidium glaucoma* increased exponentially at the beginning, but after a few days, the population densities started to drastically oscillate. These oscillations were most pronounced during the transient algae–bacteria imbalance situation between Days 40 to 60 (Fig. 2), when algal numbers were minimal. Bacterial abundance decreased after the introduction of the ciliate, but recovered slowly to higher than initial cell numbers at Day 51. The peak of bacterial abundances in the I-Stage between Days 40 to 60 was also reflected in II-Cyc.

Bacterial and protistan biomass and phosphorus (Tables 1 & 2)

Bacteria in I-Stage had the smallest mean cell volume in comparison with all other variants. Therefore, bacte-

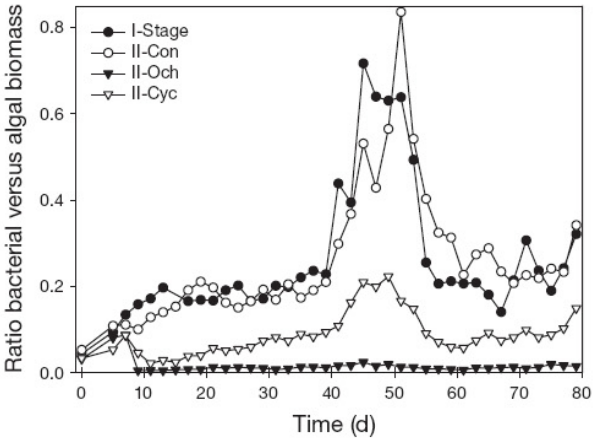

Fig. 2. Bacterial versus algal biomass ratio in the 4 vessels of the continuous cultivation system. I-Stage: *Cryptomonas phaseolus* and accompanying bacteria; II-Con: control; II-Och: *Ochromonas* sp.; II-Cyc: *Cyclidium glaucoma*

Table 1. Community parameters of bacteria, algae and predators. Values are averages calculated after the introduction of the predators (t = 68 d, n = 35). Asterisks indicate a significant difference (1-way ANOVA, $p < 0.001$) of the vessels II-Och and II-Cyc compared to vessel II-Con (control). Bac: bacteria; Alg: algae; Pre: predators (*Ochromonas* sp. in II-Och, *Cyclidium glaucoma* in II-Cyc); Σ: sum of all parameters; ALF-968, CAU663, BET-42a, BET3-446, CF1-853, HGC-69a: fluorescence *in situ* hybridization (FISH) probes

Community parameter		I-Stage	II-Con	II-Och			II-Cyc
				Total	Single cells	Microcolonies	
Abundance (cells ml^{-1})	10^6 Bac	9.90	10.00	0.44*	0.32*	0.12*	3.44*
	10^4 Alg	2.55	3.39	3.05*	–	–	4.25
	10^4 Pre	–	–	5.97	–	–	0.20
Biovolume (mm^3 l^{-1})	Bac	1.153	1.882	0.081*	0.043*	0.038*	0.777*
	Alg	9.53	14.15	13.45	–	–	17.51*
	Pre	–	–	5.42	–	–	2.27
	Σ	10.69	16.03	18.96	–	–	20.56
Biomass (µg C l^{-1})	Bac	344	518	22*	12*	10*	208*
	Alg	1432	2111	2000	–	–	2615*
	Pre	–	–	886	–	–	318
	Σ	1776	2629	2908	–	–	3141
Phosphorus[a] (µg P l^{-1})	Bac	19.8	20.1	0.9*	0.6*	0.2*	6.9*
	Alg	23.0	30.5	27.4	–	–	38.3*
	Pre	–	–	18.1	–	–	4.8
	Σ	42.8	50. 6	46.4	–	–	50.0
Bacterial assemblage composition (% of DAPI)	ALF-968	5.6	7.6	13.2	–	–	27.3*
	CAU663	2.5	3.0	8.6*	–	–	4.4
	BET-42a	86.5	81.9	43.9	–	–	44.8*
	BET3-446	41.4	37.2	20.8*	–	–	19.2*
	CF1-853	2.9	4.6	19.1*	–	–	14.5
	HGC-69a	0.5	0.7	1.6	–	–	0.8

[a]Phosphorus content of the predators was evaluated as mentioned in the text

Table 2. Single-cell characteristics of bacteria, algae and predators. Values are averages calculated after the introduction of the predators (t = 68 d, n = 35). Asterisks indicate a significant difference in single-cell characteristics (1-way ANOVA, $p < 0.001$) of the vessels II-Och and II-Cyc compared to vessel II-Con (control). Bac: bacteria; Alg: algae; Pre: predators (*Ochromonas* sp. in II-Och, *Cyclidium glaucoma* in II-Cyc); MCL: mean cell length; MCW: mean cell width; MCV: mean cell volume; CCC: cellular carbon content

Cellular parameter		I-Stage	II-Con	II-Och			II-Cyc
				Total	Single cells	Microcolonies	
MCL (µm)	Bac	1.47	1.83	–	1.43*	3.49*	1.46*
	Alg	13.42	13.92	13.92	–	–	13.68
	Pre	–	–	5.49	–	–	17.52
MCW (µm)	Bac	0.34	0.39	–	0.37*	0.35*	0.47*
	Alg	7.29	7.53	7.76	–	–	7.51
	Pre	–	–	5.49	–	–	10.92
MCV (µm^3)	Bac	0.118	0.190	-	0.135*	0.322*	0.229*
	Alg	381	422	448	–	–	413
	Pre	–	–	90	–	–	1126
CCC (fg C cell^{-1})	Bac	34.9	51.6	–	38.0*	81.6*	60.3
	10^3 Alg	57.1	62.9	66.6	–	–	61.6
	10^3 Pre	–	–	2.7	–	–	158.0

rial biomass was about 1 third lower than in II-Con, although bacterial abundances in these 2 vessels were nearly equal. In II-Och, we distinguished between freely dispersed bacterial cells and microcolonies (Table 2). Single cells quickly became significantly shorter than in II-Con, whereas cells in microcolonies were always significantly longer. Although the abundance of single cells was twice as high as of cells in microcolonies, total bacterial biomass of these 2 cell types was approximately equal. Bacterial widths were significantly larger in II-Cyc

than in the control vessel (Table 2); the same holds true for bacterial cell volumes. Bacteria in this treatment had the highest cellular carbon content and formed relatively high biomasses. Algal phosphorus content of the second stage was highest in the vessel containing the ciliate (II-Cyc) and lowest in the vessel II-Och, where the flagellate might have incorporated phosphorus due to its mixotrophic life strategy.

Changes of the bacterial assemblage due to grazing

In II-Och, only a few freely dispersed cells were present. The remaining bacteria (up to 56% of total numbers) formed microcolonies with mean numbers of 5 to 7 cells per colony, large aggregates (containing more than 25 cells per aggregate) and long, single filaments. While microcolony forming cells could be counted easily due to their loose cell contact, this was not possible for densely packed aggregates. Therefore, only single cells were evaluated by FISH. Microcolonies also occurred in stage II-Cyc, but at a later timepoint, in lower densities and with other cell morphologies. The proportion of single cells was much higher in this vessel (II-Cyc).

FISH was performed for selected sampling dates that reflected drastic population changes of either bacteria or predators (see Fig. 1). The first 2 timepoints (Day 0 and Day 9) describe the situation before and shortly after inoculation of the 2 predators. The 3 subsequent timepoints (Days 25, 35 and 49) mirror the slight (II-Och) or pronounced (II-Cyc) recovery of bacterial abundances and the eventual peak of bacterial numbers in I-Stage and II-Con. During this period, predators reached very high abundances. The sixth datapoint describes the situation of bacterial abundance decrease while predators remained stable (Day 61). The last timepoint describes the community composition at the very end of the experiment, when bacterial numbers recovered in I-Stage, II-Con, and II-Cyc (Day 79).

Approximately 95% of DAPI-stained cells could be visualized at several timepoints in hybridizations with probe EUB I-III (data not shown). Slightly lower detection rates (87%) were found in II-Och at the last sampling date. Nearly 100% of total bacterial abundance could be accounted for by the different probes (Fig. 3). At our level of analysis, the composition of the bacterial assemblages remained remarkably stable in I-Stage and II-Con even during large shifts in algal to bacterial biomass ratios. β-Proteobacteria formed the dominating group in these vessels (56 to 93% of DAPI-stained cells). About 50% of β-Proteobacteria could be assigned to '*Aquabacterium* sp.' by probe BET3-446.

In II-Och, β-Proteobacteria decreased strongly, and constituted less than 30% of bacteria in this treatment

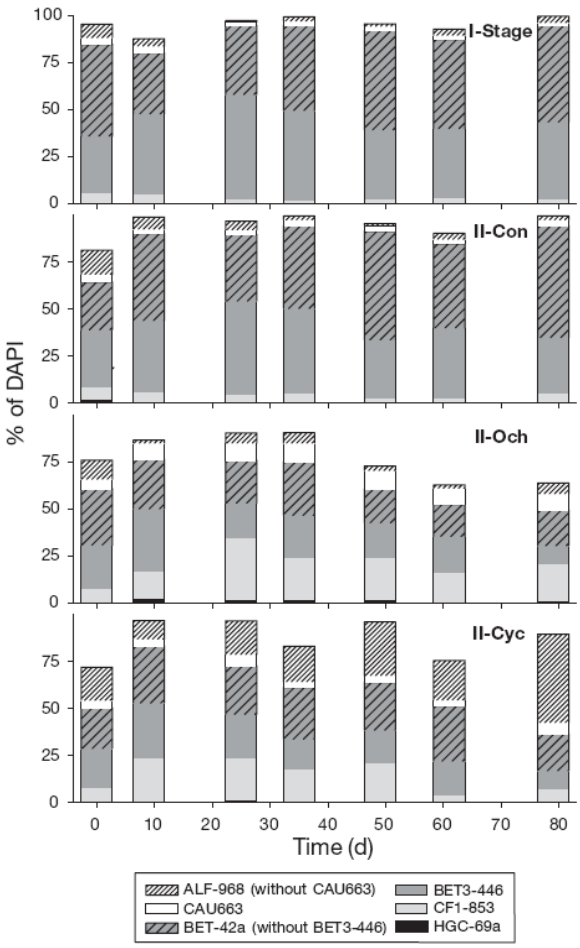

Fig. 3. Relative contribution of different bacterial groups to the assemblages, as determined by fluorescence *in situ* hybridization (FISH). Probes: ALF-968 (α-Proteobacteria without *Caulobacter* spp.), CAU663 (*Caulobacter* spp.), BET-42a (β-Proteobacteria without '*Aquabacterium* sp.'), BET3-446 ('*Aquabacterium* sp.'), CF1-853 (members of the *Flexibacter sancti–Flavobacterium ferrugineum* lineage), HGC-69a (*Actinobacteria*). Abbreviations as in Fig. 1 legend

at the end of the experiment (Figs. 3 & 4). Bacteria of the *Flexibacter sancti–Flavobacterium ferrugineum* lineage of *Sphingobacteriales* (Probe CF1-853) increased from 7% to a maximum of 32% of DAPI-stained cells at Day 25 (Fig. 3). Both single cells and microcolonies with loose cell contact were detected with this probe, and were counted separately (Fig. 5). The abundance of single cells decreased slightly thereafter, whereas the abundance of cells in microcolonies remained high. *Caulobacter*-like bacteria hybridized by Probe CAU663 increased in proportion in II-Och (maximum, 10% at Day 35), whereas other α-Proteobacteria decreased slightly.

In II-Cyc we observed a strong relative decrease in the numbers of all β-Proteobacteria and of '*Aquabacterium* sp.' (Fig. 3). Bacteria of the *Flexibacter sancti–Flavobacterium ferrugineum* lineage strongly increased until Day 49 (up to 21%) (Fig. 5), but then dropped by almost 1 order of magnitude at Day 61. At the end of the experiment, a slight recovery was observed. Bacteria hybridized with probe CAU663 were rare and remained rather stable, but other α-Proteobacteria strongly increased to 53% at the end of

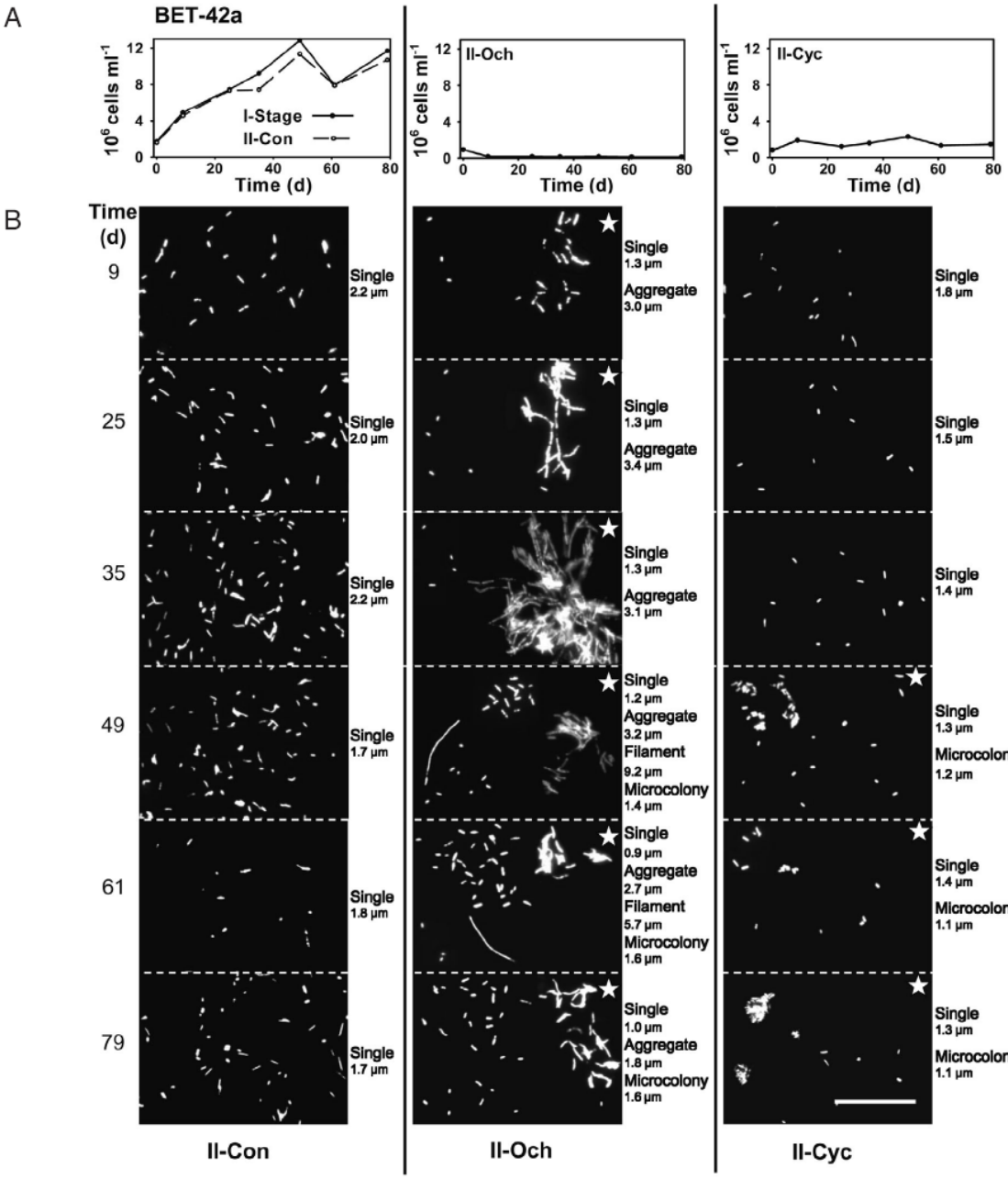

Fig. 4. Succession in (A) abundance and (B) morphotype of bacterial populations detected by probe BET-42a in the vessels II-Con, II-Och and II-Cyc. On the right side of each picture in (B) is a short description of the cell types and the mean cell lengths. White stars in II-Och and II-Cyc indicate that pictures do not represent real abundances of bacteria, but are composed of different photographs in order to show diverse morphotypes. The scale bar at the bottom is 10 µm and applies to all pictures. Abbreviations as in Fig. 1 legend

the experiment (Fig. 6). In this treatment, a succession of bacterial populations was observed that contrasted with the development of these populations in I-Stage and II-Con: a decline of β-Proteobacteria and a rapid increase of bacteria from the *Flexibacter sancti–Flavobacterium ferrugineum* lineage was followed by a strong decrease of the latter group and a bloom of α-Proteobacteria.

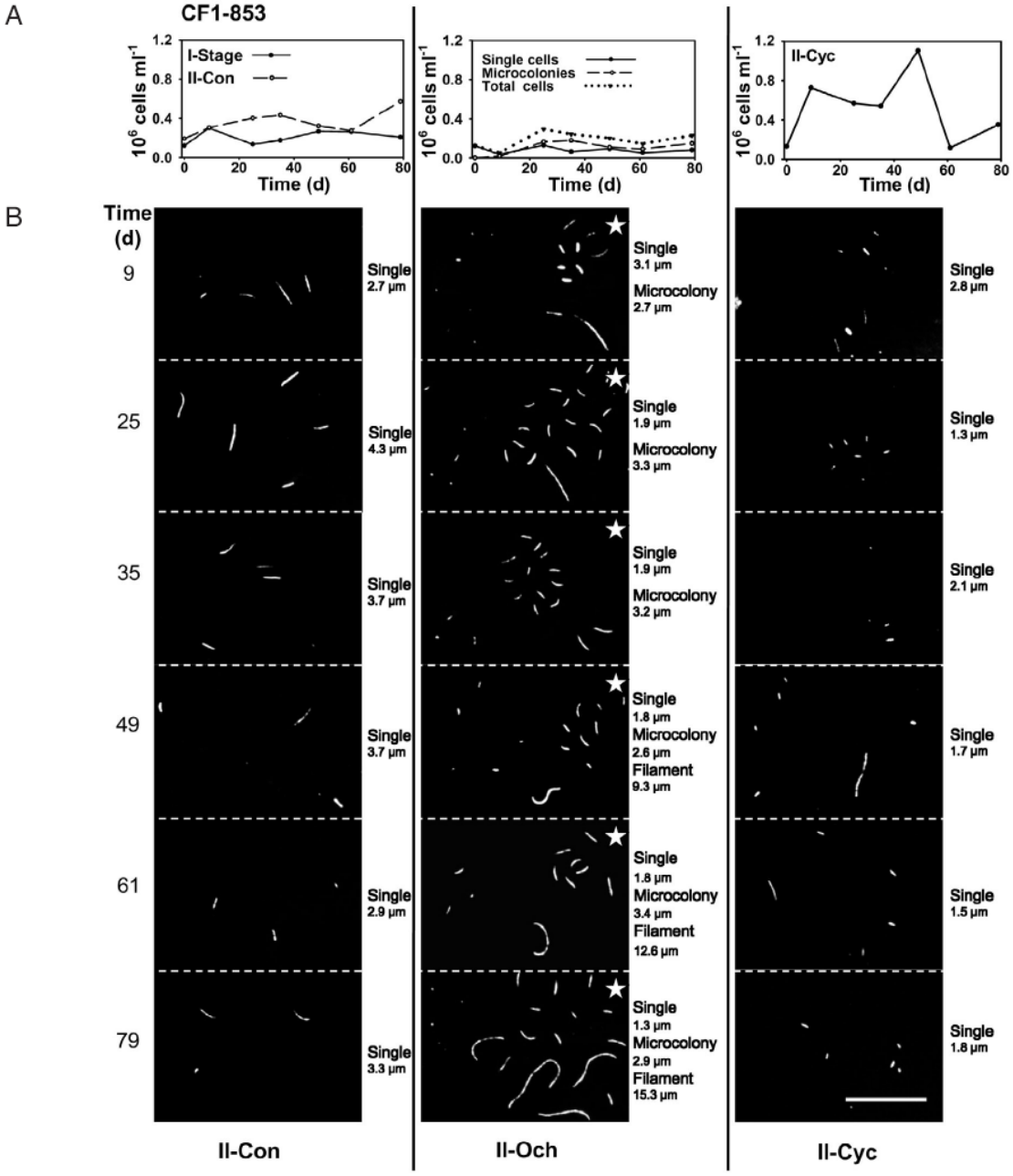

Fig. 5. Succession in (A) abundance and (B) morphotype of the bacterial populations detected by probe CF1-853 in the vessels II-Con, II-Och and II-Cyc. On the right side of each picture in (B) is a short description of the cell types and the mean cell lengths. White stars in II-Och indicate that pictures do not represent real abundances of bacteria but are composed of different photographs in order to show diverse morphotypes. The scale bar at the bottom is 10 µm and applies to all pictures. Abbreviations as in Fig. 1 legend

Morphological shift of the different bacterial groups

β-Proteobacteria (Fig. 4) showed a high phenotypic plasticity, and 5 different morphotypes could be observed. In I-Stage and II-Con, these bacteria formed medium-sized single rods, whereas in II-Och, all single cells were smaller. Immediately after inoculation of *Ochromonas* sp., large aggregates occurred, which

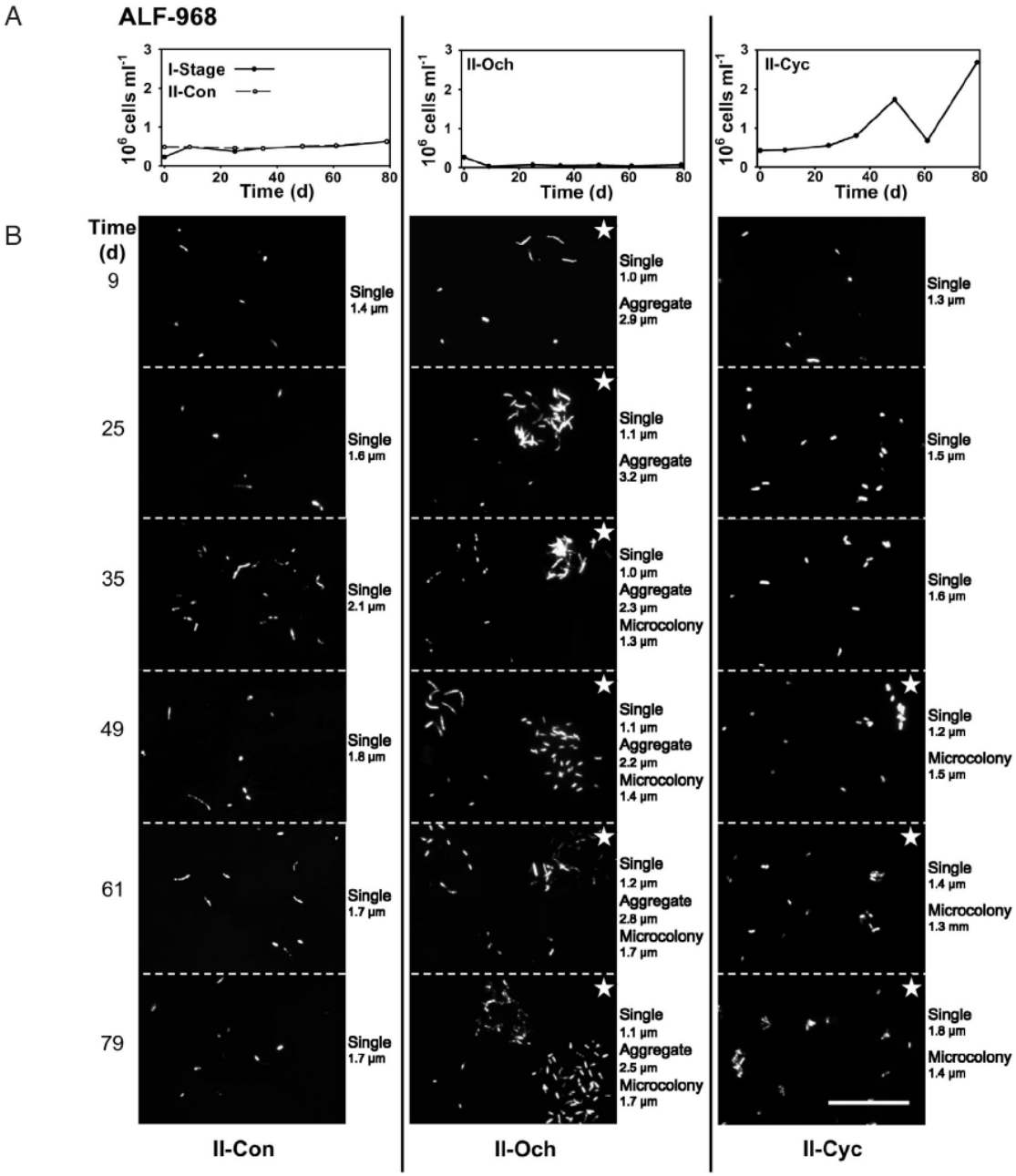

Fig. 6. Succession in (A) abundance and (B) morphotype of the bacterial populations detected by probe ALF-968 in the vessels II-Con, II-Och and II-Cyc. On the right side of each picture in (B) is a short description of the cell types and the mean cell lengths. White stars in II-Och and II-Cyc indicate that pictures do not represent real abundances of bacteria but are composed of different photographs in order to show diverse morphotypes. The scale bar at the bottom is 10 µm and applies to all pictures. Abbreviations as in Fig. 1 legend

contained primarily bacteria related to *Aquabacterium* sp. (probe BET3-446), with some cells affiliating with *Caulobacter* spp. (probe CAU663). These aggregates were not very abundant, but became very large (more than 25 cells per aggregate). At Day 49, 2 more morphotypes of β-Proteobacteria were discernable: long rare filaments and small microcolonies with loose cell contact, which increased in numbers until the end of the experiment. These 2 morphotypes could not be detected with probe BET3-446. All β-Proteobacteria in II-Cyc formed rod-shaped single cells until Day 49. Afterwards, microcolonies with tight cell contact appeared, which became very abundant. Cells in these colonies could not be detected with probe BET3-446.

Bacteria related to the *Flexibacter sancti–Flavobacterium ferrugineum* lineage (Fig. 5) formed single rods in I-Stage and II-Con. In II-Och, there was a morphological separation between small single cells and microcolonies containing 5 to 7 larger cells with loose cell contact. These colonies became dominant during the experiment (up to 53% in comparison to abundances of single cells). At Day 49, a third cell type hybridizing with CF1-853 appeared, consisting of long filaments, which slightly increased in abundance until the end of the experiment. In the ciliate vessel (II-Cyc), only 1 cell type was hybridized by probe CF1-853, i.e. small rod-shaped single cells.

α-Proteobacteria (Fig. 6) fell into a large range of size classes in I-Stage and II-Con, from small coccoid forms to rod-shaped bacteria. In II-Och, shortly after inoculation of *Ochromonas* sp., *Caulobacter* spp. was present in aggregates dominated by '*Aquabacterium* sp.' (see above). The remaining α-Proteobacteria formed very small single cells until Day 35. At this timepoint small colonies of α-Proteobacteria with loose cell contact appeared, which became more abundant towards the end of the experiment. In the vessel containing *Cyclidium glaucoma,* α-Proteobacteria formed rod-shaped single cells. At Day 49, small colonies appeared also in II-Cyc, but these colonies had direct cell contact, and were not hybridized by probe CAU663.

DISCUSSION

Effects of predators with differing feeding strategies

Ochromonas sp. reached very high abundances shortly after inoculation and minimized bacterial abundance dramatically (Fig. 1). In contrast to former studies on the same experimental community no bloom of *Actinobacteria* was observed in II-Och (Pernthaler et al. 2001, T. Posch et al. unpubl. data). However, we still found a clear relationship between grazing pressure and bacterial size structure. Shortly after introduction of the predator, the bacterial assemblage responded by a bidirectional shift towards small single cells and very large and complex morphologies, such as aggregates, microcolonies and filaments. This grazing-protective strategy has also been found in previous studies (Güde et al. 1989, Pernthaler et al. 1996, Hahn & Höfle 1999, Jürgens & Matz 2002). *Ochromonas* sp. preferentially feeds on bacteria larger than 0.2 μm^3 (~0.7 µm in diameter, Andersson et al. 1986). Although there was a shift towards smaller cells, 78% of all free-living bacterial cells found in II-Och were within the edible size range (>0.2 μm^3), which probably explains why these cells did not recover in abundance. On the one hand, morphological changes could be a consequence of size selectivity by the flagellate. On the other hand, the observed phenomena could also be caused by the extremely high total grazing rates irrespective of size selectivity. Due to its mixotrophic life strategy, *Ochromonas* sp. can maintain high densities, thus exerting high grazing pressure on bacteria even when only little particulate food is available. When grown under light, the flagellate increasingly uses its ability to grow photosynthetically, if bacterial densities fall below a concentration of $<10^6$ cells ml^{-1} (Rothhaupt 1996b, 1997), and it has to take up phosphate. As a consequence, bacteria in II-Och had to compete for phosphorus not only with the algae (Mindl et al. 2005), but also with a predominantly phototrophic bacterivore. Another effect of the phosphorus-incorporation of the flagellate is that algae had the lowest phosphorus content in this vessel.

In contrast, exclusively heterotrophic protists such as *Cyclidium glaucoma* release surplus phosphorus and other nutrients, and therefore stimulate P-limited algal and bacterial growth (Eccleston-Parry & Leadbeater 1995, Rothhaupt 1997). This beneficial effect was found in II-Cyc, where algae reached higher densities than in all other vessels. Interestingly, bacteria grazed by the filter feeder *C. glaucoma* reacted with an increase in cell width rather than in cell length (Table 2). This phenomenon was also observed in earlier chemostat experiments (Posch et al. 2001). Considering the complex structures of the ciliate feeding apparatus, it is conceivable that such increased cell width might provide at least partial protection against grazing by *C. glaucoma*.

Effects on assemblage composition (Table 3)

The ability to form grazing-resistant morphologies seems to be widespread among various bacterial phylotypes (Table 3). Field studies as well as laboratory experiments typically show that freshwater β-Proteobacteria are negatively affected by protistan grazing if

Table 3. Examples for the effects of protistan grazing on the bacterial assemblage composition observed in (a) field and (b) chemostat studies applying fluorescence *in situ* hybridization (FISH) (only examples for freshwater systems). Abbreviations for oligonucleotide probes: *Bacteria:* EUB = EUB338; *Actinobacteria*: Ac1 = Ac1-847, HGC = HGC-69a; *Archaea*: ARCH = ARCH915; α-Proteobacteria: ALF = ALF-968, CAU = CAU663, PS = *Pseudomonas* sp.; β-Proteobacteria: BET = BET-42a, BONE = BONE23a; BET3 = BET3-446, R-BT = R-BT65; γ-Proteobacteria: GAM = GAM-42a; *Cytophaga–Flavobacterium–Bacteroides*: CF = CF-319a, CF1 = CF1-853, R-FL = R-FL615. FRACT = size fractionation, LONG = longitudinal transect, TRANS = transplant experiment

(a)

Lake	Applied probes	EUB (%)	Natural assemblages	Effects of grazing	Time/ Notes	Source
Small fishless pond	EUB HGC ALF1b BET BONE GAM CF	60–85	BET > CF	Decrease in BET & CF Increase in ALF & GAM Mainly ALF formed filaments	0–140 h Data from Daphnia-free variants	(1)
Rímov reservoir	EUB ALF BET GAM CF	45–85	BET ~ CF > ALF > GAM	Increase in ALF & CF ALF formed filaments	0–96 h FRACT, clear water phase	(2)
		45–60	BET > CF > ALF > GAM	Decrease in BET increase in ALF & CF CF formed filaments	0–96 h FRACT, late phytoplankton peak	(2)
Three small fishless ponds	EUB ARCH ALF1b			Aggregates dominated by ALF, freely dispersed cells dominated by BET	Data from Daphnia-free variants	(3)
Rixdorf	BET GAM CF	70–95	BET ~ CF	Increase in BET & ALF Decrease in CF	0–94 h	
Freudenholm		60–80	BET > CF > ALF	Increase in BET & ARCH Decrease in CF	0–116 h	
Hasselburg		50–80	BET > CF > ALF ~ ARCH	Increase in ALF	0–94 h	
Sau Reservoir	EUB ALF BET GAM CF	60–90	CF > BET > ALF ~ GAM Biomass dominance of BET	Decrease in BET & CF	April, July 1997 LONG	(4)
Rímov reservoir	EUB ALF BET R-BT GAM CF R-FL	75–85	BET > CF > GAM > ALF	Increase in BET & CF R-FL formed filaments Aggregates by BET & CF	0–96 h FRACT	(5)
Rímov reservoir	EUB ALF BET R-BT GAM CF R-FL	50–85	BET~CF>R-BT>GAM	Decrease in BET & R-BT	0–96 h FRACT TRANS	(6)

Table continued on next page

they morphologically resemble cocci or small single rods (Jürgens et al. 1999, Posch et al. 2001, Šimek et al. 2001b, Gasol et al. 2002). Via food vacuole inspection, we found that 85% of all ciliates and 23% of all flagellates had ingested β-Proteobacteria. This hints at an unresolved paradox within aquatic microbial communities, since this phylogentic group (together with *Actinobacteria*) forms a prominent fraction in many freshwater systems (Methé & Zehr 1999, Glöckner et al. 2000, Burkert et al. 2003). However, at present only few groups of the β-Proteobacteria are known to be strongly affected by grazing, e.g. '*Aquabacterium* sp.' (Pernthaler et al. 2001) and members of the freshwater beta I clade (Šimek et al. 2001b).

Table 3 (continued)

(b) Chemostat predators	Applied probes	EUB (%)	Natural assemblages	Effects of grazing	Time/ Notes	Source
Bodo saltans	EUB ALF BET GAM CF	80–97	BET > ALF ~ GAM > CF	Decrease in BET Increase in ALF	12 d	(7)
Ochromonas sp.	EUB PS BET GAM	81	GAM > BET > PS	Increase in flocs (PS)	261 d	(8)
Cyclidium glaucoma, *Bodo saltans*, *Ochromonas* sp.	Ac1 CAU BET3	–	BET3 ~ CF > CAU > Ac1	Dominance of Ac1 Increase in ALF & CF1 Decrease in BET3	12–16 d	(9)
Cyclidium glaucoma	ALF BET CF	–	ALF > BET > CF	Decrease in BET & CF	16d	(10)

(1) Jürgens et al. (1999), (2) Šimek et al. (1999), (3) Langenheder & Jürgens (2001), (4) Šimek et al. (2001a), (5) Šimek et al. (2001b), (6) Šimek et al. (2003), (7) Šimek et al. (1997), (8) Hahn & Höfle (1999), (9) Pernthaler et al. (2001), (10) Posch et al. (2001)

Some pelagic freshwater β-Proteobacteria might be able to rapidly increase their growth rates upon environmental change (Jürgens et al. 1999, Šimek et al. 2001b, Burkert et al. 2003). Other species of β-Proteobacteria potentially exhibit high phenotypic plasticity, i.e. they can form various morphotypes such as microcolonies, filaments and aggregates (Jürgens et al. 1999, Šimek et al. 2001b, Hahn et al. 2004). In our system, such large and complex structures were obviously resistant to protistan grazing, as they were not found in protists food vacuoles and maintained relatively high abundances. β-Proteobacteria related to the drinking-water biofilm bacterium *Aquabacterium* sp. (Kalmbach et al. 1999) were able to form morphotypes that were resistant even to strong grazing (Fig. 4). They constituted 46 ± 8% of all β-Proteobacteria in I-Stage, II-Con and II-Cyc, and this proportion remained stable throughout the experiment with slightly lower values for II-Och (42 ± 7%). These bacteria formed large branched colonies consisting of at least 25 cells in the vessel containing the flagellate. They are phylogenetically closely related to stream biofilm bacteria (Brümmer et al. 2003), and to a freshwater isolate (MWH55) that was found to develop microcolonies when exposed to grazing by *Ochromonas* sp. strain DS (Hahn et al. 2004). In our experiment, this extreme response to grazing pressure was only observed in II-Och but not in II-Cyc, where grazing rates were much lower. At present, we can only speculate if this phenomenon is linked to the contrasting size selectivity of the 2 predators, or if it is due to different remineralization rates of the mixotrophic and the heterotrophic protist.

Members of the α-Proteobacteria usually do not form a dominant fraction of freshwater bacterioplankton (Methé & Zahr 1999, Glöckner et al. 2000, Klammer et al. 2002). Interestingly, these bacteria were favored in many investigations that feature an experimental increase in grazing pressure (Table 3, Jürgens et al. 1999, Šimek et al. 1999). Bacteria hybridized by probe CAU663 contributed 48 ± 18% of all α-Proteobacteria in I-Stage and II-Con, with higher values for II-Och (68 ± 18%) and lower values for II-Cyc (18 ± 7%). The typical long stalks of single *Caulobacter* spp. cells (Poindexter et al. 2000) might have caused difficulties especially for the filter-feeding ciliate which only rarely ingested bacteria detected by probe CAU663. Members of α-Proteobacteria seemed to be generally less vulnerable to grazing by *Cyclidium glaucoma*, although they were also found in the ciliate's food vacuoles. However, a variety of morphotypes related to α-Proteobacteria dominated the bacterial assemblage in II-Cyc at the end of the experiment. Again, it seems to be a paradox that members of α-Proteobacteria can coexist with strong grazing pressure but are rare in freshwaters. This suggests that factors other than protistan grazing also substantially affect the abundance of particular bacterial genotypes in freshwater systems.

Apart from morphological changes of single taxonomic groups we could also observe bacterial coexis-

tence in aggregates as a result of strong grazing pressure. The large branched colonies formed by members of the BET3-446 cluster were always associated with single cells affiliated with *Caulobacter* spp. A similar phenomenon has been described from samples of a meso-eutrophic reservoir, where large aggregates of β-Proteobacteria were interspersed with members of the *Sphingobacteriales* (Šimek et al. 2001b). Thus, protistan grazing does not only directly affect individual phylogentic clusters, but may also modify the interactions between different taxonomic groups.

Bacteria of the *Flexibacter sancti–Flavobacterium ferrugineum* lineage of *Sphingobacteriales* also exhibited a wide spectrum of morphotypes. Interestingly, microcolonies and long filaments only occurred in the presence of *Ochromonas* sp., but not with *Cyclidium glaucoma* (Fig. 5). This again illustrates that microbial predation-defence mechanisms may be triggered only by particular predator species. Since the probe is targeted to a lineage harbouring a number of distinct phylotypes (Pernthaler et al. 2001), the presence of both filaments and microcolonies may either reflect the phenotypic plasticity of a single population, or of co-occurring bacterial populations affiliated with this phylogenetic clade.

Successions in mixed microbial assemblages

It is likely that microbial assemblages respond differently to gradual and to rapid changes of grazing-induced mortality. In most experimental studies, 'unnaturally' strong shifts of grazing pressure are induced, either by inoculating high numbers of protistan predators or by decoupling the microbial food web by size fractionations (Table 3). Such experimental set-ups will lead to rather extreme responses, which probably occur in nature only during particular time periods. In contrast, it is not well understood how a mixed bacterial assemblage is affected by varying levels of protistan grazing pressure, e.g. throughout the season. Freshwater pelagic bacteria are typically confronted with changing intensities of grazing pressure, as well as with shifts in the species composition of the dominant bacterivorous protists (Sanders et al. 1989, Sommaruga & Psenner 1995, Cleven & Weisse 2001). Therefore, it is likely that seasonal community patterns of bacterivorous protists are reflected in successions of bacterial phylotypes. In addition, these successions will also be determined by bottom–up factors, e.g. the availability of limiting substrates and nutrients.

Unfortunately, it is hardly feasible to experimentally mimic protistan species successions. However, it is possible to examine if there might be successions of mixed bacterial assemblages if exposed to different grazing modes over an extended period of time. In our experiment, we attempted to model a microbial community which features some of the complexity of natural planktonic systems, where interactions between bacteria, algae, and bacterivorous protists occur simultaneously. We hypothesized that there might be no stable long-term response of the bacterial assemblage in such a complex system even if the physico-chemical parameters were tightly controlled. This was verified both by the more pronounced genotypic shifts in the grazing exposed assemblages (Fig. 3), and the successions of different grazing resistant morphotypes (Figs. 4 to 6).

Another very basic interaction that may introduce instability both in our system and in the freshwater pelagic zone is the competition for limiting phosphorus. The repeatedly observed oscillation of bacterial versus algal biomass ratios in the cultivation system (Fig. 2, Mindl et al. 2005) is most likely caused by this phenomenon. According to the so-called phytoplankton–bacteria paradox (Bratbak & Thingstad 1985), the ratio of bacterial versus algal biomass increases with decreasing availability of phosphorus. In previous chemostat experiments with media containing >40 $\mu g\,P\,l^{-1}\,d^{-1}$ (Mindl et al. 2005), the ratio of bacterial versus algal biomass ranged around 0.05 to 0.1. These ratios remained stable over periods of 2 mo. In contrast, the use of media with 20 $\mu g\,P\,l^{-1}\,d^{-1}$ led to a bacteria:algae ratio of 0.2 to 0.3, followed by considerable oscillations (Fig. 2 and Mindl et al. 2005). A similar situation may occur in natural oligo- to mesotrophic freshwater systems. Since bacterial carbon supply in the first stage of our system exclusively depends on exudates of *Cryptomonas phaseolus*, these imbalance situations only lasted for several days.

Implications for future studies

Presently it is still poorly understood which bacterial phylotypes in freshwaters are preferentially eliminated by bacterivores, and how the development of different grazing protection mechanisms by specific bacterial populations is affected by the availability of nutrients. Future studies are required to explore how (1) the intensity of grazing, (2) the community composition and (3) the size selectivity of protistan grazers will influence the phenotypes and the seasonal dynamics of different bacterial populations in pelagic systems.

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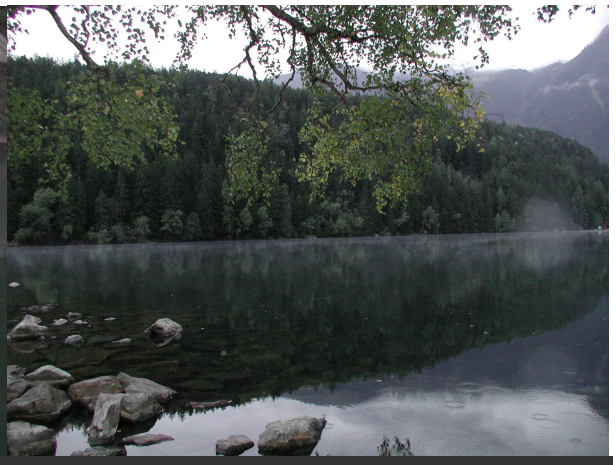

paper II

Modulation of microbial predator-prey dynamics by phosphorus availability. Growth patterns and survival strategies of bacterial phylogenetic clades

Salcher MM, Hofer J, Hornak K, Jezbera J, Sonntag B, Vrba J, Simek K, Posch T

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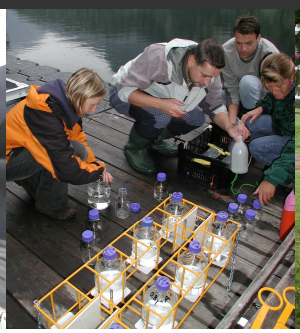


Modulation of microbial predator–prey dynamics by phosphorus availability: Growth patterns and survival strategies of bacterial phylogenetic clades

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Abstract

We simultaneously studied the impact of top-down (protistan grazing) and bottom-up (phosphorus availability) factors on the numbers and biomasses of bacteria from various phylogenetic lineages, and on their growth and activity parameters in the oligo-mesotrophic Piburger See, Austria. Enhanced grazing resulted in decreased proportions of bacteria with high nucleic acid content (high-NA bacteria) and lower detection rates by FISH. There was a change in the composition of the bacterial assemblage, whereby *Betaproteobacteria* were heavily grazed while *Alphaproteobacteria* and *Cytophaga–Flavobacterium–Bacteroides* were less affected by predators. Changes in bacterial assemblage composition were also apparent in the treatments enriched with phosphorus, and even more pronounced in the incubations in dialysis tubes (allowing relatively free nutrient exchange). Here, *Betaproteobacteria* became dominant and appeared to act as successful opportunistic competitors for nutrients. In contrast, *Actinobacteria* did not respond to surplus phosphorus by population growth, and, moreover, maintained their small size, which resulted in a very low biomass contribution. In addition, significant relationships between high-NA bacteria and several bacterial phylogenetic clades were found, indicating an enhanced activity status. By combining several single-cell methods, new insight is gained into the competitive abilities of freshwater bacteria from a variety of phylogenetic lineages under contrasting sets of bottom-up and top-down constraints.

Introduction

Grazing by bacterivorous protists and nutrient availability are known to be the major limiting factors faced by bacterial assemblages in freshwater habitats. The interplay of these factors has been the subject of a multitude of experimental studies during the recent decades (for reviews, see Sanders *et al.*, 1989; Jürgens & Güde, 1994). Laboratory studies (e.g. batch cultures or chemostat experiments) and field experiments have provided new insight into the general rules of the interaction between different taxonomic groups of bacteria and their protistan predators (Horňák *et al.*, 2005; Jezbera *et al.*, 2005; Pernthaler, 2005; Salcher *et al.*, 2005; Šimek *et al.*, 2005).

To elucidate bottom-up effects on bacterial phylogenetic lineages, several manipulation studies have been carried out by enrichment with a variety of nutrients (e.g. Pérez & Sommaruga, 2006) or by transplanting bacterial assemblages from nutrient-poor to nutrient-rich habitats (Gasol *et al.*, 2002; Šimek *et al.*, 2006) or vice versa (Gasol *et al.*, 2002; Horňák *et al.*, 2005).

Datasets from lakes with different trophic status point to a relationship between the response of bacterial assemblages to bottom-up and top-down manipulations and the ambient nutrient levels. Oligotrophic systems are thought to be more bottom-up-controlled, while top-down pressure plays just a minor role (Sanders *et al.*, 1992). The opposite seems to apply for eutrophic lakes, where grazing by different trophic levels is assumed to be the major limiting factor for bacterial development. However, the effects of predation on bacterial assemblages can be buffered by high nutrient availability, and consequently by rapid growth of the

prominent bacterial lineages (Šimek *et al.*, 2003, 2005). Hence, opportunistic bacteria with high growth rates can establish high standing stocks even though their numbers are heavily reduced by grazers. Moreover, bacteria could also be stimulated as a result of nutrient recycling by grazers (Pernthaler *et al.*, 1997; Jezbera *et al.*, 2006).

It is known, however, that certain bacteria can resist high grazing pressure. Associated strategies include morphological adaptations that allow the bacteria to escape the optimal prey-size spectrum of the predators, i.e. shifts towards smaller or larger cell sizes or the formation of inedible aggregates or microcolonies (for reviews see Hahn & Höfle, 2001; Jürgens & Matz, 2002; Pernthaler, 2005). Bacteria with high phenotypic plasticity can be found in a wide range of phylogenetic clades (e.g. within *Cytophaga–Flavobacterium–Bacteroides* or several subgroups of *Proteobacteria*), but the underlying mechanisms (e.g. triggering of filament–formation) are still controversial (Hahn *et al.*, 1999; Corno & Jürgens, 2006).

A further aspect of protistan grazing is its pronounced impact on the activity status of bacterial assemblages. Several studies have shown that protistan predators selectively feed on active bacteria (Del Giorgo & Gasol, 1995; Posch *et al.*, 1999; Šimek *et al.*, 2003; Hornák *et al.*, 2005). Therefore, high grazing pressure may often lead to a decrease in bacterial bulk activity. However, grazing can also have a beneficial effect on bacterial activity owing to nutrient recycling (Jürgens & Sala, 2000; Sherr & Sherr, 2002).

The activity status of bacteria can be followed indirectly by the ratio of bacteria with high to those with low nucleic acid content (high NA vs. low NA), as revealed by means of flow cytometry (Gasol *et al.*, 1999), or by the amount of ribosomes that can be determined with FISH techniques (EUB detection rate, Oda *et al.*, 2000).

We present here a size-fractionation and nutrient-enrichment experiment carried out in the oligo-mesotrophic Piburger See, Austria. We intended to deepen our knowledge about the underlying factors shaping the dynamics of bacterial clades present in the lake by simultaneous bottom-up and top-down manipulations. Our aims were to elucidate: (i) which bacterial groups provided the opportunistic competitors for the uptake of surplus phosphorus (P); (ii) which clades were able to form grazing-resistant morphologies under different nutrient regimes; (iii) the impact of grazing and nutrients on the activity status of bacteria; and (iv) the synergistic effects of top-down and bottom-up factors.

Materials and methods

Study site and set-up of the experiment

The experiment was carried out at the oligo-mesotrophic Piburger See, Austria (for details, see Tolotti & Thies, 2002). Lake water from 0.5 m depth was filtered by means of two stainless steel filtration devices. Two size fractions of lake-water microbial assemblages were produced by sequential filtration of raw water through filters (Osmonics) with pore sizes of 5 μm or 0.8 μm. Duplicate set-ups (2 L each) of these fractions were incubated in (i) bottles (2-L Schott glass bottles, termed < 5 μm Bottle or < 0.8 μm Bottle), (ii) bottles enriched with *c.* three-fold *in situ* phosphorus (P) concentration (15 μg P L^{-1}; termed < 5 μm Bottle+P or < 0.8 μm Bottle+P), or (iii) prerinsed dialysis tubes (Spectra-Por, 12 000–14 000 molecular weight cut-off termed < 5 μm Dialysis or < 0.8 μm Dialysis). All variants were incubated in the lake for four days and subsamples of 400 mL were taken daily.

Abundance of microorganisms, flagellate grazing rates

Twenty-millilitre subsamples were fixed with formaldehyde (3% final concentration), and organisms were stained with DAPI (Porter & Feig, 1980), filtered on black polycarbonate filters (0.22-μm pore size, Osmonics), and further processed as described in Posch *et al.* (1999).

We used fluorescently labelled bacteria (FLB) to determine flagellate grazing rates (Šimek *et al.*, 1999). Briefly, we added FLB (*c.* 18% of actual bacterial concentration) to 50-mL unfixed subsamples of each < 5 μm treatment for 10–20 min. After fixation with the alkaline Lugol's solution – formaldehyde – thiosulfate decolorization technique (Sherr *et al.*, 1987), 10–30 mL were stained with DAPI and filtered onto black polycarbonate filters (1-μm pore size, Osmonics). At least 50 flagellates were counted and inspected for FLB ingestion by means of epifluorescence microscopy (Zeiss Axioplan). Average FLB uptake rates were multiplied by flagellate abundance to estimate total grazing rates (TGR) of each treatment.

Flow cytometry

Duplicate 10-mL samples were fixed with paraformaldehyde (1% final concentration, pH 7.4) and stained with the nucleic acid stain SYTO 13 (Molecular probes, Eugene, OR) at a final concentration of 2.5 μM. In addition, Triton-X 100 (0.1% final concentration) and fluorescent microspheres (1 μm TransFluoSpheres 488/560, Molecular Probes) as a counting and internal fluorescence reference were added. Flow-cytometric analyses were performed on a MoFlo (DakoCytomation, Glostrup, Denmark) equipped with a water-cooled argon ion 4 W Innova 90 C1 laser (Coherent, Santa Clara, CA) tuned to 488 nm with an output power of 200 mW. The orthogonal side scatter (SSC) was measured at 488/10 nm, the green fluorescence of SYTO 13 at 530/40 nm, and the yellow signals from the microspheres at 570/40 nm. Detectors were R-1477

photomultiplier tubes (Hamamatsu, Hamamatsu City, Japan) at 540, 470, and 570 V for SSC, SYTO 13, and yellow signals, respectively. Measurements were triggered on logarithmically amplified SYTO 13 or yellow signals using a logical OR on a custom multiple trigger board (DakoCytomation). The heterotrophic bacterial community was discriminated by manual gates from background caused by electronic noise, microspheres, and protists. Likewise, gates were drawn to distinguish between bacteria with high and low NA content. These discriminations were clearly visible, and identical gates were applied to all samples.

FISH and catalyzed reporter deposition (CARD-FISH)

Subsamples of 10–15 mL were fixed with buffered paraformaldehyde (2% final concentration, pH 7.4), filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2-μm pore size, 47-mm diameter), and stored at $-20\,^{\circ}C$ until further processing. CARD-FISH was carried out as previously described by Sekar *et al.* (2003) using the following horseradish peroxidase-labelled probes: EUB I-III for all *Bacteria* (Daims *et al.*, 1999); the probes ALF968 (Neef, 1997) and BET42a (Manz *et al.*, 1992) for the *Alpha* and *Beta* subgroups of *Proteobacteria*, respectively; probe CF319 (*Cytophaga-Flavobacterium-Bacteroides*, Manz *et al.*, 1996); the actinobacterial probe HGC69a (Roller *et al.*, 1994); and probe R-BT065 (Šimek *et al.*, 2001), which detects a subcluster within *Betaproteobacteria*. Signal-amplification was carried out with fluorescein isothiocyanate (FITC)-labelled tyramides. For counting and determination of biomasses, a Zeiss Axiophot epifluorescence microscope was used. Cell dimensions of all FISH-positive FITC-labelled bacterial cells within the studied bacterial phylogenetic lineages were measured by image analysis from images at blue excitation (488-nm wavelength). As the dimensions of CARD-FISH-stained bacteria overestimate the cell size in comparison with DAPI-stained cells, we show only the percentage of biomass contribution of each group to the total biomass of cells hybridized with probe EUB.

Growth rates of the various bacterial clades in the < 0.8-μm variants were calculated from the abundances at two consecutive sampling dates, assuming exponential growth.

Results

Bacterial abundance

Bacterial counts acquired by direct counting with epifluorescence microscopy were highly significantly correlated with values gained by flow cytometry ($r^2 = 0.93$, $P < 0.001$). Bacterial abundance passed through significant changes during the experiment (Table 1). While abundances increased significantly in all < 0.8-μm treatments and in the < 5-μm dialysis variant, the opposite was true for the < 5-μm bottle treatments. More detailed data of bacterial abundances, biomasses and stoichiometry can be found in Posch *et al.* (submitted).

Table 1. Flagellate abundances (10^6 cells L^{-1}) and total grazing rates (10^7 bacteria $L^{-1}\,h^{-1}$) of the < 5-μm variants at times 0 and 96 h Bacterial abundances (10^9 cells L^{-1}) and proportions of the various bacterial clades (EUB in % of DAPI; ALF, BET, ACT, CFB, and R-BT in % of EUB and % of EUB biomass) of all variants at times 0 and 96 h. Each value is a mean of two replicates

	< 0.8-μm variants				< 5-μm variants			
	96 h				96h			
	0 h	Bottle	Bottle+P	Dialysis	0 h	Bottle	Bottle+P	Dialysis
HNF abundance (10^6 cells L^{-1})†	–	–	–	–	1.41	11.54***	11.44***	6.30***
Total grazing rate (10^7 bacteria $L^{-1}\,h^{-1}$)†	–	–	–	–	2.64	21.30*	35.52***	12.29**
Bacterial abundance (10^9 cells L^{-1})†	3.23	5.17**	7.10***	9.72***	3.47	3.45	3.76	10.34**
EUB (% of DAPI)	70.3	81.0	86.3*	84.5*	74.4	53.9**	41.0***	78.0
ALF (% of EUB)	1.8	2.2	5.1	11.9***	1.9	8.7***	12.0***	15.1*
BET (% of EUB)	10.3	23.4***	36.9***	57.2***	10.6	16.8	21.0	59.4**
ACT (% of EUB)	33.0	20.3	30.0	23.7	31.8	37.8	42.4	16.9*
CFB (% of EUB)	1.7	2.1	4.9***	6.4**	2.0	8.3*	12.4***	7.3**
R-BT065 (% of EUB)	4.8	10.7	10.1	15.5	4.8	10.3	27.0	28.2
Sum of general probes (without R-BT065)	46.8	48.0	76.9	99.2	46.3	71.6	87.8	98.7
ALF (% of EUB biomass)	6.4	7.0	4.6	11.7	10.1	15.0	21.6*	31.2*
BET (% of EUB biomass)	27.4	42.0	51.0*	84.6	41.0	17.2*	44.4	70.0
ACT (% of EUB biomass)	12.7	7.5	4.1	4.0	14.6	8.3	7.2	2.9*
CFB (% of EUB biomass)	7.7	5.7	4.1**	4.8	9.6	19.0	15.0*	10.1

Asterisks indicate significant differences between 0 and 96 h (one-way ANOVA with Turkey's HSD posthoc test:
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
†Data from Posch et al. (submitted).

Abundance of heterotrophic nanoflagellates, and total grazing rates

Decoupling of flagellates from higher trophic levels resulted in accelerated growth rates and significant increases of heterotrophic nanoflagellates (HNF) and their bacterivory in all three < 5-μm variants (Table 1, Fig. 1a). The highest increase was found in < 5 μm Bottle+P, followed by < 5 μm Bottle, while in dialysis tubes HNF abundance and total grazing rates showed just a slight rise after four days. Overall, bacterial abundances were negatively correlated with total grazing rates ($r^2 = 0.70$, $P < 0.001$).

Changes in bacterial activity parameters

In the grazing-free (< 0.8-μm) fractions, both EUB detection rates and proportions of high-NA bacteria increased, but to different extents (Fig. 1b–g). While EUB-positive cells increased slightly in all treatments, there was a significant shift from initially dominant low-NA bacteria to high-NA bacteria within bottles+P and dialysis tubes. In the bottle treatments without P enrichment, high- and low-NA bacteria remained in stable proportions throughout the experiment.

In the < 5-μm bottle treatments, EUB detection rates decreased significantly and were negatively correlated with total grazing rates ($r^2 = 0.58$, $P < 0.001$). Within dialysis tubes, where the lowest grazing rates were observed, EUB detection rates increased slightly, while in the lake, detection remained constant ($68.4 \pm 4.9\%$). In the presence of grazers, the fraction of high-NA bacteria reacted differently depending on the nutrient manipulation. It either stayed stable (bottles), went back to approximately initial values after a slight increase (bottle+P), or increased until the end of the experiment (dialysis tubes).

Changes in bacterial assemblage composition

We detected 38–99% (mean: 61%) of EUB-positive bacteria with the four general probes ALF968, BET42a, CF319a, and HGC69a. In lake-water samples, all bacterial groups showed stable percentages throughout the investigation period.

In the absence of grazers, *Betaproteobacteria* (BET) were the most prominent group, with two-fold higher percentages at the last date than initally (Table 1, Fig. 2), and, therein, the R-BT065 subcluster of BET showed nearly the same increase ($\times 2.2$). There was a clear effect of the P-addition on members of *Cytophaga—Flavobacterium–Bacteroides* (CFB: $\times 2.9$), *Alphaproteobacteria* (ALF: $\times 2.8$), and BET ($\times 3.6$), which increased, whereas *Actinobacteria* (ACT) maintained constant values. This effect of surplus nutrients was even more pronounced in the dialysis tubes, where ALF and BET showed almost six-fold higher proportions at the end of the experiment, CFB increased by four-fold, while ACT decreased ($\times 0.7$).

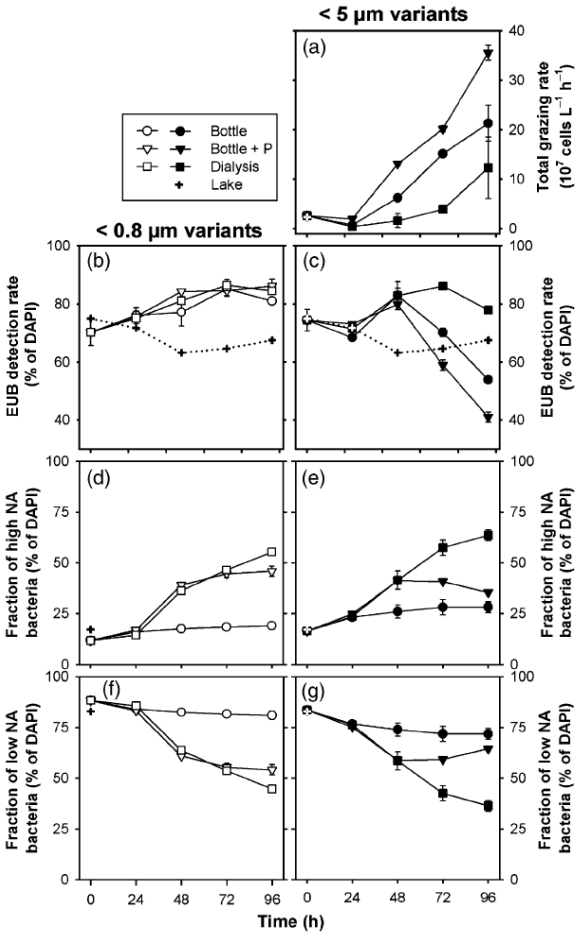

Fig. 1. (a): Total grazing rates (10^7 cells L^{-1} h^{-1}); (b, c): EUB detection rates (% of DAPI); (d, e): proportions of high-NA bacteria (% of DAPI); (f, g): proportions of low-NA bacteria (% of DAPI). (a, c, e, g): Data from < 5-μm variants (with HNF); (b, d, f): data from < 0.8-μm variants (without HNF). Values are the means of two replicate set-ups, and whiskers show the SE of these values.

In the < 5-μm treatments (Table 1, Fig. 2), the percentages of ALF and CFB increased more than four-fold, while BET and ACT remained stable. The addition of P also stimulated growth of ALF ($\times 6.5$) and CFB ($\times 6.2$) in these variants, but not of BET nor of ACT. In the dialysis-tube treatments, BET dominated the assemblages, although ALF showed the highest rise ($\times 8.1$). CFB did not respond to high nutrient concentrations, while ACT declined to 50% of their original abundance.

Contribution of bacterial phylogenetic lineages to total bacterial biomass

While ACT accounted for about 29% of total bacterial abundance, on average they contributed only 7.7% to

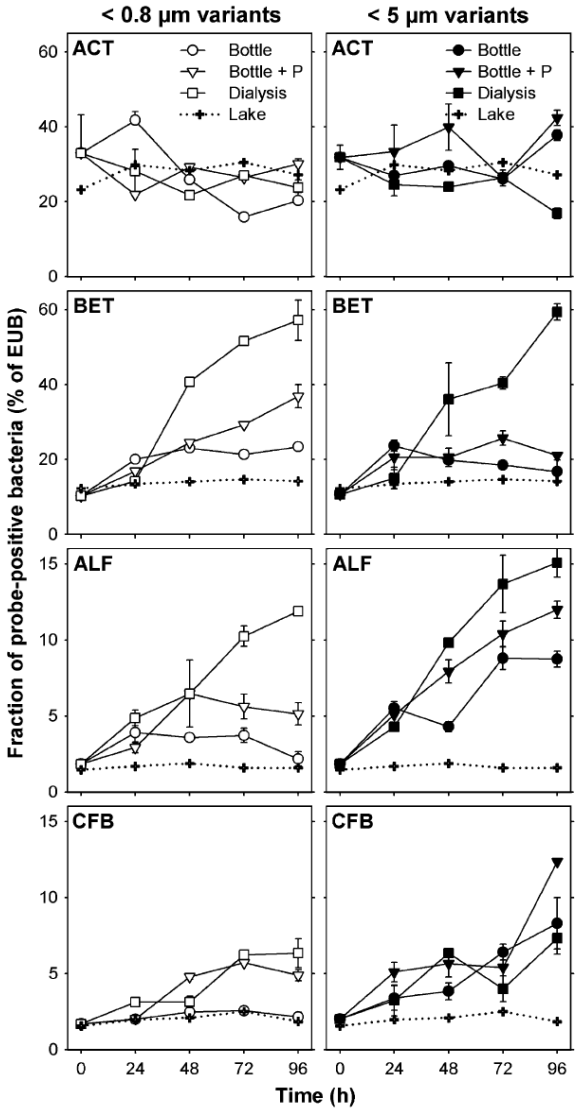

Fig. 2. Proportions of the bacterial clades *Actinobacteria* (ACT), *Betaproteobacteria* (BET), *Alphaproteobacteria* (ALF), and *Cytophaga–Flavobacterium–Bacteriodes* (CFB) as a percentage of EUB-positive cells. Values are the means of two replicate set-ups, and whiskers show the SE of these values.

bacterial biomass (Table 1). These bacteria were always very small and of uniform vibrioid morphology. The opposite was found for nearly all other bacterial groups: their contribution to EUB biomass was disproportionately higher than their contribution to total abundance. This was most apparent for ALF and CFB in the $<$ 5-µm variants (more than two-fold higher values for biomasses), whereas in the $<$ 0.8-µm variants such phenotypic adaptations were found only for BET (nearly two-fold higher values for biomasses). ALF comprised filamentous strains in all $<$ 5-µm variants and in $<$ 0.8-µm Dialysis, but not in the $<$ 0.8-µm bottle treatments, whereas CFB formed filaments only in the dialysis-tube treatments, regardless of grazing pressure.

Growth rates of the bacterial groups

We found highly contrasting patterns of growth rates of the bacterial clades in the $<$ 0.8-µm fractions (Fig. 3). While net growth rates in the lake ranged around 0, uncoupling of the food web by filtration resulted in significantly higher values.

EUB-positive and DAPI-positive bacteria featured similar values, whereas high-NA bacteria always had higher, and low-NA bacteria had much lower growth rates. Moreover, group-specific growth rates were higher compared with total bacteria and eubacteria. ALF, BET and CFB showed almost the same growth patterns, while growth of ACT was delayed for about 1 day compared with the other groups.

In the bottle treatments, growth rates declined very quickly, although it was mainly ALF and BET that benefitted from the absence of predators during the first 2 days. At the end of the experiment, only ACT showed positive growth rates, even though these bacteria had exhibited negative values the days before, while growth of ALF and CFB ceased.

This 'bottle effect' was partially buffered in the bottles+P, where very high growth rates during the first 2 days were followed by a sharp decline. Here, high-NA bacteria showed their highest growth rates between days 2 and 3 (corresponding to a net doubling time of 13 h), which were quite similar to the growth rates of ALF, BET and CFB (corresponding to net doubling times of 13, 17, and 12 h, respectively).

In the dialysis tubes, all bacterial groups profited from continuous nutrient supply. BET grew fastest (corresponding to a doubling time of 11 h) and showed a high similarity to growth-rate patterns of high-NA bacteria. Again, ACT exhibited a delay of one day in their growth rate responses.

Relationships between high-NA bacteria and bacterial phylogenetic lineages determined by CARD-FISH

Abundances of high-NA bacteria correlated with most of the bacterial phylogenetic lineages (Fig. 4), while low-NA bacteria showed only a weak positive correlation with EUB abundances ($r^2 = 0.62$, data not shown). There were highly significant correlations with EUB, ALF, BET and CFB (Fig. 4). However, the highest correlation was found by comparing the sum of the latter 3 groups (ALF, BET, CFB) with high-NA bacteria ($r^2 = 0.95$). Interestingly, the slope of the regression line corresponded almost exactly to the 1 : 1 line. ACT was the only bacterial group that showed no significant positive correlation with either high- or low-NA bacteria.

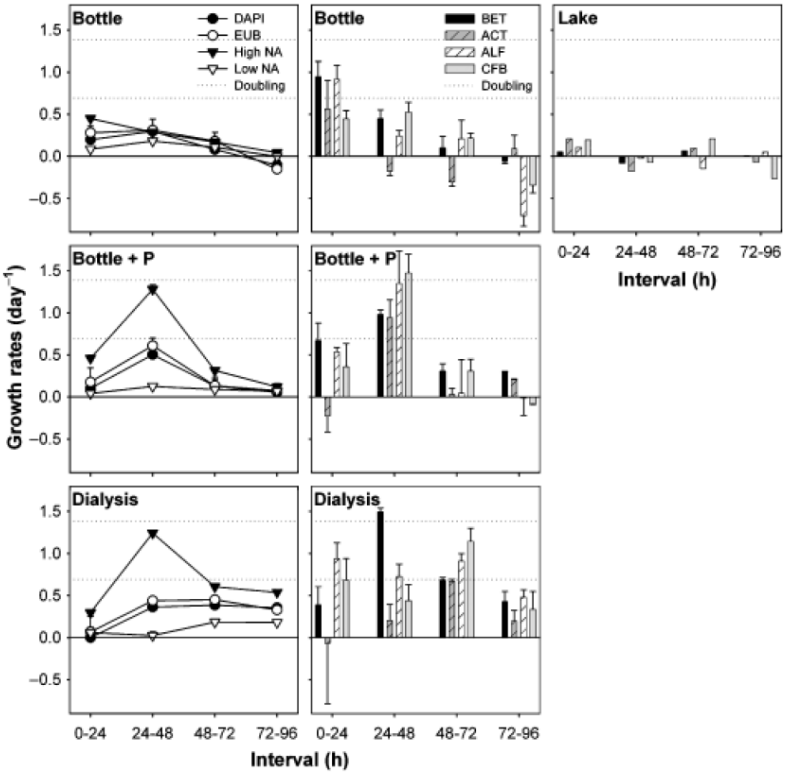

Fig. 3. Growth rates (d^{-1}) of the bacterial clades in the < 0.8-μm variants and the lake. Abbreviations as in Figs 1 and 2. Lines show the growth rates for one and two duplications per day.

Discussion

Activity status determined by flow cytometry and CARD-FISH

Very often, scatter plots of aquatic prokaryotes labelled with DNA stains gained by flow cytometry show two distinct subgroups: the high-NA fraction is assumed to be the proportion of 'active' bacteria (Gasol *et al.*, 1999), while bacteria with a low nucleic acid content seem to be more or less inactive. This assumption was confirmed by several cell sorting studies, where high-NA bacteria had higher amino acid uptake and growth rates (Servais *et al.*, 1999; Lebaron *et al.*, 2002; Longnecker *et al.*, 2006; Zubkov *et al.*, 2006). Moreover, a transplant and grazing experiment in a eutrophic reservoir (Gasol *et al.*, 2002) revealed a significant correlation between the fraction of high-NA bacteria and EUB detection rates, as was also found in our experiment (Fig. 4). In the above-mentioned study, the abundance of EUB-positive bacteria vs. high-NA bacteria fitted almost the 1 : 1 line, while this was not the case in our study. This could be explained by the different methodologies used, because Gasol and *et al.* (2002) applied FISH with fluorescently monolabelled probes (Alfreider *et al.*, 1996; Glöckner *et al.*, 1996) to determine bacterial assemblage composition. Owing to the higher detection rates of CARD-FISH, the intercept of the regression line between high-NA and EUB in our study was higher than 1 (Fig. 4). The best relationship was found between high-NA bacteria and the sum of bacteria hybridized with probes ALF, BET and CFB ($r^2 = 0.95$), with a regression line that fitted nearly exactly the 1 : 1 line. The exclusion of ACT from the regression could make sense, if their small cell size and morphology is interpreted as inactivity and a low nucleic acid content. However, the opposite effect was found by Warnecke *et al.* (2005), who detected high DNA *de novo* synthesis rates of ACT in various mountain lakes. Moreover, MAR-FISH (microautoradiography combined with FISH) investigation of ACT in a high mountain lake revealed a high leucine uptake activity (Pérez & Sommaruga, 2006). Similarly, Nielsen *et al.* (2006) found higher thymidine uptake activity among ACT than among all *Bacteria* in a drinking-water reservoir. In our study, abundances of ACT were also not correlated with low-NA bacteria (data not shown), and ACT showed positive growth rates (Fig. 3), which can be regarded as additional proof that they were not inactive or dormant in the lake.

Do different bacteria from large phylogenetic lineages form coherent functional guilds?

Until recently, aquatic bacteria were seen as a 'black box', and their ecological role was studied regardless of their

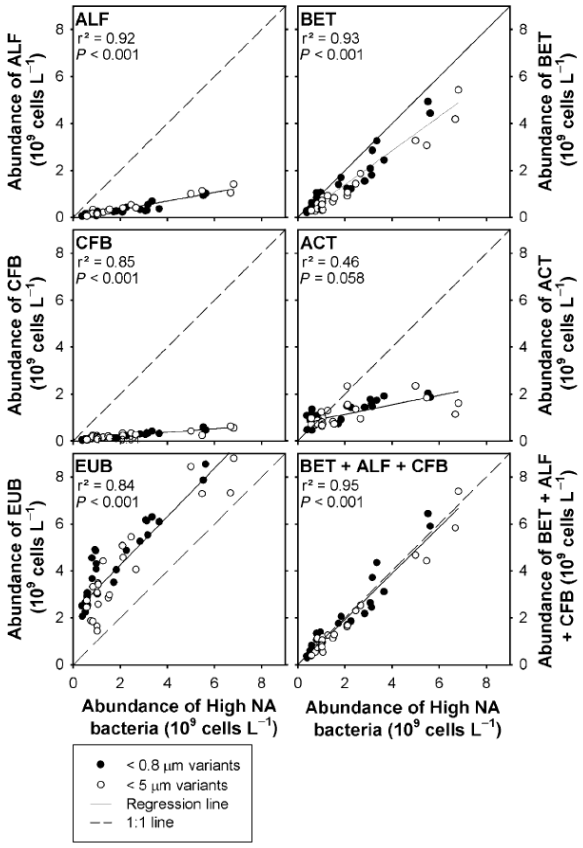

Fig. 4. Correlations between high-NA bacteria (10^9 cells L^{-1}) and the various bacterial clades (10^9 cells L^{-1}). Abbreviations as in Fig. 2.

phylogenetic affiliation. In this context, Pedrós-Alió (1989) suggested the introduction of 'functional guilds'; that is, even if the taxonomic background of certain bacterial species is not known, they could still be grouped together in terms of their physiology and ecological function. Moreover, one could even claim that such functional guilds could not only be classified physiologically, but also morphologically or even phylogenetically. To date, little is known about the physiological features of abundant freshwater bacterial clades, because most of them have not yet been cultivated and are known only from 16S (or 23S) rRNA gene sequence data. Moreover, it is problematic that FISH probes for large phylogenetic groups often cover physiologically and ecologically very diverse microorganisms (e.g. CFB). More specific probes are rarely used because they detect only a small fraction of total bacterial abundances.

Our results support the assumption that BET follow 'opportunistic' growth strategies; that is, they are favoured under limited grazer control and good nutrient availability, but are heavily grazed when environmental conditions change towards the dominance of top-down control. This phylogenetic group plays a very important role in freshwater ecosystems owing to its high abundance and biomass. However, in several lakes ACT are the most abundant group (Warnecke *et al.*, 2005), although, in terms of biomass, BET can still account for the largest part in bacterioplankton assemblages (Table 1). Several strains of the prominent *Polynucleobacter necessarius* cluster (also called beta II cluster) have recently been isolated, with very small cell sizes and distinct morphologies (Hahn, 2003). This group and another very abundant group of the beta I cluster (belonging to the *Rhodoferax* sp. BAL47 cluster, detectable with probe R-BT065, Šimek *et al.*, 2001) were able to overgrow other bacterial groups within a few days and become dominant in the absence of predators (Šimek *et al.*, 2001; Burkert *et al.*, 2003; Pérez & Sommaruga, 2006). This was also found in our experiment, in which BET accounted for 60% of all bacteria within the dialysis tubes, but to only 17% in the bottles experiencing the most intense HNF bacterivory (Fig. 2). Moreover, bacteria belonging to the narrower phylogenetic linage of the R-BT065 cluster accounted for up to 50% of all BET on the last day (Table 1), while in the lake they remained stable at around 22% (data not shown).

There was a clear difference between the < 0.8-μm bottle+P and the dialysis treatments, in which bacteria were able to incorporate nutrients from surrounding water (Fig. 2), and therefore it seems that BET were not exclusively limited by P. In a dissolved organic matter (DOM)-enrichment experiment conducted in a high mountain lake, BET and especially the R-BT065 subcluster showed high abundances and activities when enriched with algal-derived DOM (Pérez & Sommaruga, 2006), whereas in an enrichment with soil-derived DOM, ACT were more efficient. On the one hand, BET (and particularly R-BT065) show very high activity and growth rates (Jürgens *et al.*, 1999; Šimek *et al.*, 2001, 2003, 2005; Gasol *et al.*, 2002; Hahn, 2003), even under high grazing pressure. On the other hand, these bacteria are preferably ingested by flagellates, as revealed with FISH-analysis of HNF food vacuoles (Jezbera *et al.*, 2005, 2006; Salcher *et al.*, 2005). In conclusion, this opportunistic growth strategy with fast physiological shift-ups could represent a certain protection against grazing by compensating for the losses with high growth rates.

ACT are very abundant in limnetic ecosystems (Glöckner *et al.*, 2000; Sekar *et al.*, 2003; Allgaier & Grossart, 2006) and can show a certain resistance to UV radiation (Warnecke *et al.*, 2004; 2005). However, it is only recently that our knowledge of their dynamics has begun to expand, with the application of the more sensitive CARD-FISH protocol (Sekar *et al.*, 2003). ACT are known to be less grazing-vulnerable because of their small size, which could be below the uptake limits of certain protists (Pernthaler *et al.*, 2001),

as recently confirmed by Jezbera *et al.* (2005, 2006) in a series of *in situ* experiments. Speculations about their inedibility include their cell-wall structure and even toxicity. Hahn *et al.* (2003) demonstrated the complete protection of an isolated strain of ACT against predation by *Ochromonas* sp. strain DS. In our experiment, ACT showed no clear trend in their abundance (Fig. 2), which is in contrast to prior experiments (Pernthaler *et al.*, 2001; Šimek *et al.*, 2001, 2005; Horňák *et al.*, 2005). However, one effect was visible: ACT were not very efficient competitors in taking up nutrients; that is, they were often outcompeted by other, rapidly growing bacterial clades such as BET. In the dialysis variants (highest nutrient availability), we found that ACT decreased, pointing to an antagonistic relationship between these two clades. The same phenomenon was described for an enrichment experiment in an acidic bog lake (Burkert *et al.*, 2003), where members of the beta II cluster outcompeted ACT within a few days. In our study, ACT growth rates were lower than those for all other groups (Fig. 3). Besides, they were of uniform, small size, and, although they formed the bulk of hybridized cells (up to 42% of EUB), their contribution to bacterial biomass was small (between 3% and 15% of EUB biomass, Table 1).

In contrast, ALF and CFB showed different patterns. In the $<$ 5-μm fraction, these bacteria increased both in abundance and in biomass (Table 1). The increase in biomass of these bacteria was about two-fold higher than that in abundance; that is, they increased not only numerically, but also in size at the same time. This can be seen as a grazing-protection mechanism and is further evidence of their high phenotypic plasticity (Jürgens *et al.*, 1999; Kirchman, 2002). In the predator-free variants, ALF showed no or just a slight increase in cell length, and CFB became even smaller (data not shown). CFB can display very high growth rates when heavily grazed by protists (Jürgens *et al.*, 1999), but slowed down when grazing pressure is eliminated experimentally (Fuchs *et al.*, 2000; Šimek *et al.*, 2003). Moreover, this clade contains strains that are able to form filaments (Hahn *et al.*, 1999; Hahn & Höfle, 2001; Pernthaler *et al.*, 2004; Salcher *et al.*, 2005) or aggregates (Šimek *et al.*, 2001) under high grazing pressure.

The same strategy seems to hold true for ALF. Several studies have shown that these bacteria are enriched when protistan grazing is high (Pernthaler *et al.*, 1997; Jürgens *et al.*, 1999; Langenheder & Jürgens, 2001; Salcher *et al.*, 2005), partly because they form filaments and aggregates together with other bacterial phyla (Langenheder & Jürgens, 2001; Salcher *et al.*, 2005). Although they are present in small proportions in lakes (2% of EUB in Piburger See) they can eventually increase if top-down pressure occurs (15% in $<$ 5-μm dialysis, Fig. 2).

Synergistic effects of bottom-up and top-down factors

The assemblage composition in our enrichment experiment does not reflect the usual composition of freshwater bacterioplankton. Specifically, it seems peculiar that grazing-resistant ALF and CFB do not dominate natural assemblages. However, HNF grazing pressure is always linked to several other factors and cannot be seen as the only influence on bacteria. Grazing by zooplankton, substrate availability, intraspecific and interspecific competition, and host-specific virus-induced mortality are of major importance for shaping bacterial assemblages.

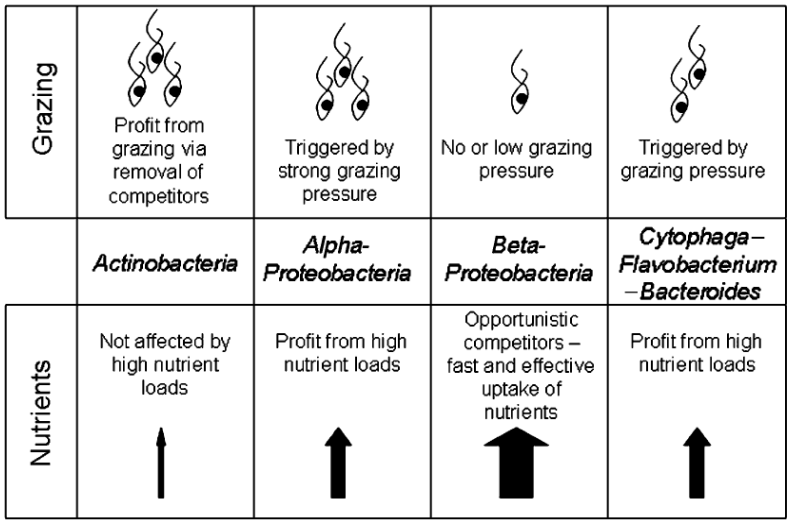

Fig. 5. Schematic depiction of the synergistic effects of bottom-up and top-down factors inducing the dominance of bacterial clades in Piburger See.

However, some experimentally observed patterns can also be found in freshwater habitats. At certain time points in the seasonal cycle (late spring bloom), bacteria allow themselves the 'luxury' of filament formation (Jürgens & Güde, 1994; Jürgens & Stolpe, 1995; Pernthaler *et al.*, 1996, 2004) but this is induced not only because of high grazing pressure but also as a result of high nutrient availability. In such situations nutrients might provide the necessary preconditions for filamentation, whereas grazing acts as a trigger. Mainly bacteria of the CFB cluster (Hahn *et al.*, 1999; Pernthaler *et al.*, 2004), but also some ALF and BET (Jürgens *et al.*, 1999; Langenheder & Jürgens, 2001) dominate these filamentous morphotypes that are less vulnerable to HNF predation. Later on, during the clear-water phase with peaking mesozooplankton, this morphology disappears because filaments are filtered out by Daphnids (Jürgens & Stolpe, 1995; Degans *et al.*, 2002; Vrba *et al.*, 2003; Pernthaler *et al.*, 2004).

Moreover, grazing by protists may not exclusively have a negative impact on bacterioplankton (Fig. 5). Some strains (e.g. ALF, CFB) might benefit from nutrients released by predators, while others (e.g. ACT) might benefit from changed competition owing to the removal of competitive but grazing-vulnerable bacteria (e.g. BET). Moreover, strains from ALF and CFB with high phenotypic plasticity could be enriched because of their inedible size (filaments, aggregates). Jürgens & Matz (2002) showed a schematic depiction of possible regulatory mechanisms for the development of grazing-resistant morphologies that includes some of the above-mentioned factors. However, in that review, bacteria were still seen as a black box; that is, their phylogenetic background was not considered. Therefore, we want to highlight that, with the application of single-cell techniques, some bacterial groups, even if they are categorized into large phylogenetic entities (i.e. ALF, BET, CFB, and ACT), are now successfully identified as being supported or repressed by at least some of the above-mentioned mechanisms (Fig. 5).

Acknowledgements

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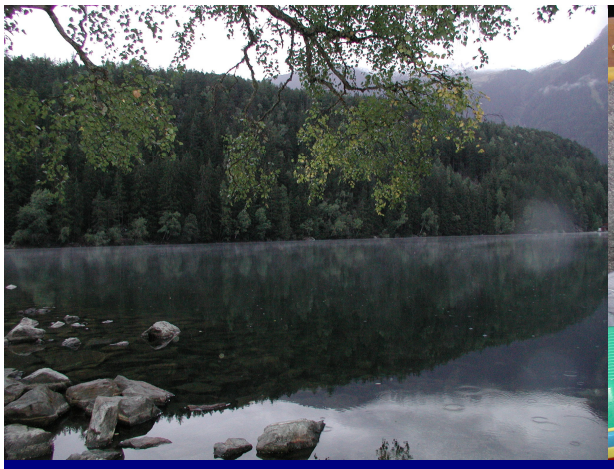

paper III

Biomass reallocation within freshwater bacterioplankton induced by manipulating phosphorus availability and grazing

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Biomass reallocation within freshwater bacterioplankton induced by manipulating phosphorus availability and grazing

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ABSTRACT: Grazing by bacterivorous protists and the availability of nutrients strongly affect the taxonomic composition of freshwater bacterioplankton. However, so far, studies have focussed mainly on changes in abundance. Therefore, we studied biomass reallocation within different phylogenetic lineages of bacteria under varying regimes of protistan grazing and nutrient supply in the oligo-mesotrophic Piburger See (Austria). Size fractionation of lake water was used to create setups with and without bacterivores. These treatments were incubated in bottles (with and without added P) and in dialysis tubes (allowing for free nutrient exchange) for 96 h. The release of bacteria from grazing resulted in a small increase in total abundance, but more pronounced changes of production and biomass. Addition of P to bottles seemed to indicate P limitation of bacterial growth. However, this was contradicted by the results of dialysis tube incubations. In these treatments, highest bacterial biomass and production were observed as well as a substantial increase in particulate P in the bacterial size fraction. While *Betaproteobacteria* abundance and biomass increased greatly in P-surplus bottles (4× and 12×, respectively), biomass increased even more in the dialysis tubes (28×). After 48 h incubation, virtually all imported P in predator-free dialysis tubes could be attributed to newly produced betaproteobacterial biomass. These bacteria were significantly reduced by the presence of predators in the bottle incubations only, probably due to the delayed growth of bacterivores in the dialysis tubes. By contrast, although *Actinobacteria* numerically dominated in the lake, as well as in the grazing-exposed bottle treatments, their contribution to total biomass was always low. Our results illustrate that the quantification of the biomass of specific lineages may allow for a better assessment of C and P fluxes within microbial food webs. Moreover, nutrient addition during bottle incubations might result in misleading conclusions about growth limitation.

KEY WORDS: *Actinobacteria* · Bacterial biomass · Bacteria–flagellate interactions · Bacterial lineages · *Betaproteobacteria* · Fluorescence *in situ* hybridization · Phosphorus enrichment

INTRODUCTION

Analysis of the bacterial assemblage composition in various freshwater systems initially suggested a numerical dominance of *Betaproteobacteria* (Alfreider et al. 1996, see also Table 1 in Bouvier & del Giorgio 2003). However, subsequent methodological improvements, namely the combination of catalyzed reporter deposition with fluorescence *in situ* hybridization (CARD-FISH), have changed this view by highlighting the important role of Gram-positive *Actinobacteria* within freshwater bacterioplankton (Sekar et al. 2003, Wu et al. 2006). Several studies have confirmed the numerical dominance of these 2 phylogenetic lineages

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in freshwater lakes (Table 6 in Klammer et al. 2002, Bouvier & del Giorgio 2003, Warnecke et al. 2005). However, most investigations have primarily focused on abundance, whereas our knowledge about the biomass allocation within freshwater phylogenetic lineages is limited (Yokokawa et al. 2004). A detailed look at the contribution of different bacteria to total biomass might substantially change our view on the importance of these groups, leading us to the understanding that less-abundant bacteria with larger cell sizes (e.g. filamentous cells) might contribute substantially to the microbial C pool (Schauer & Hahn 2005).

The proportion of bacteria from different phylogenetic groups is regulated by top-down (grazing) and bottom-up (nutrient availability) controls (Horňák et al. 2005). While our knowledge about the role of nutrients for the success of distinct lineages is still restricted to single observations (Pérez & Sommaruga 2006, Šimek et al. 2006), the structuring impact of bacterivores on freshwater assemblages has been elucidated in detail (see Table 3 in Salcher et al. 2005). *Betaproteobacteria* are often selectively eliminated by grazing protists (Jezbera et al. 2006), whereas *Actinobacteria* maintain or even increase their relative proportions at high grazing pressure. This holds true for both field experiments and laboratory-based experimental assemblages, and it has partially been explained as a consequence of size-selective protistan feeding (Pernthaler et al. 2001, Hahn et al. 2003). Since bacterivorous protists selectively consume larger bacterial morphotypes, they will affect not only cell numbers, but also (and to a greater extent) the biomass ratios of different freshwater lineages. Moreover, grazing is not only a key factor in directly shaping bacterial assemblages, but also an intrinsic step in the remineralization of nutrients (Rothhaupt 1997). As a consequence, grazing might also modify the competition within the bacterioplankton assemblage in cases of strong nutrient limitation (Salcher et al. 2007).

We analyzed the biomass reallocation between freshwater bacterial lineages in the context of P availability and grazing pressure. Raw water of the oligo-mesotrophic Piburger See (Austria) was size fractionated to generate variants consisting of bacteria only or of bacteria and their major consumers, heterotrophic nanoflagellates (HNF). We chose 2 approaches to manipulate P availability: (1) the incubation of samples in non-penetrable bottles with and without additional P under *in situ* conditions; and (2) the *in situ* exposure of filtrates in dialysis tubes, allowing for the relatively free exchange of nutrients. In these setups we investigated the effects of P availability and grazing on the biomass of 4 prominent phylogenetic lineages in the context of bacterial production and P partitioning between bacteria and HNF (see also Salcher et al. 2007).

MATERIALS AND METHODS

Study site. Piburger See (Austria) is a small prealpine lake which was strongly affected by anthropogenic-induced eutrophication during the 1960s and 1970s. For the restoration of the lake, in 1970 a tube was permanently installed at the 24 m depth to continuously remove oxygen-depleted and P-rich hypolimnetic water. Although the oxygen saturation in deeper water subsequently improved, it took nearly 30 yr to decrease P concentrations (Tolotti & Thies 2002). Current annual averages of the epi-, meta-, and hypolimnetic water strata are around 6, 7 and 9 µg P l^{-1}, respectively. At the time of sampling (September 2003) the major limnological variables (at 0.5 m depth) were as follows: temperature = 18.5°C, oxygen = 8.8 mg l^{-1}, chlorophyll *a* (chl *a*) = 3.8 µg l^{-1}, dissolved organic carbon (DOC) = 2.4 mg l^{-1}.

Experimental setup. Lake water (50 l) from 0.5 m depth was transported in 25 l bottles to the laboratory. We produced 2 size fractions by sequential filtration of the raw water samples. Size fractionations were conducted with 2 autoclaved stainless steel filtration devices (140 mm diameter). Gravity filtration over 5 µm pore-size polycarbonate filters (Osmonics) generated a filtrate consisting of bacteria and their major predators, i.e. mostly HNF. Half of the 5 µm filtered sample was subsequently filtered through 0.8 µm pore-size polycarbonate filters using a peristaltic pump in order to remove the bacterivores. The different filtrates were incubated *in situ* (at 0.5 m depth) for 4 d in setups of the following design: 2 l Schott glass bottles served as controls (referred to as <0.8 or <5 µm Bottle) for treatments (bottles) enriched with 15 µg l^{-1} of P (K_2HPO_4), assigned as <0.8 or <5 µm Bottle & P. In a third variant, 2 l of each size fraction were incubated in pre-rinsed dialysis tubes (assigned as <0.8 or <5 µm Dialysis; Spectra-Por, 12 000–14 000 molecular weight cut off). Each variant was set up in duplicate, i.e. in sum 8 bottles and 4 dialysis bags were incubated in the lake. Subsamples of 400 ml were taken each morning (08:00 h) for the analyses described below.

Abundance and biomass of the total bacterial assemblage. Twenty ml of the subsamples were fixed with 0.2 µm prefiltered formaldehyde (2% final concentration). Organisms were stained with DAPI (5 to 10 µg ml^{-1} final concentration, Porter & Feig 1980), filtered on black polycarbonate filters (0.22 µm pore-size, Osmonics) and further processed as described in Posch et al. (1997). We used the image analysis software LUCIA (Laboratory Imaging Prague) for the size determination of organisms. At least 500 bacteria per sample were measured and bacterial mean cell volumes (MCV_{BAC} in μm^3) were converted to cellular C contents (CC_{BAC} in fg C $bacterium^{-1}$) applying the formula

$CC_{BAC} = 218 \times MCV_{BAC}^{0.86}$ (Loferer-Krößbacher et al. 1998). Since this equation was developed using bacterioplankton data from Piburger See, a high precision of this volume-to-C conversion factor can be presumed (see also Posch et al. 2001). Bacterial biomass (in µg C l^{-1}) was calculated by multiplying CC_{BAC} by bacterial abundance (in bacteria l^{-1}).

HNF numbers and feeding rates. HNF grazing was estimated using fluorescently labeled bacterioplankton (FLB) following the protocol of Šimek et al. (1999). HNF cell-specific FLB uptake rates were multiplied with HNF abundance to estimate total grazing rates (TGR) of each treatment, used for the calculation of bacterial net production rates (see below). All methodological details on the quantification of HNF are described in Salcher et al. (2007).

Bacterial production. Triplicate samples of 5 to 10 ml of raw water were incubated with [methyl-^{3}H]thymidine (5 nM final concentration, 83 Ci $mmol^{-1}$ specific activity, Amersham) for 1.5 h at *in situ* temperatures. For each treatment 2 formaldehyde-fixed control samples were incubated under the same conditions. After fixation with formaldehyde (2% final concentration) samples were filtered onto white polycarbonate filters (0.2 µm pore-size, 25 mm diameter, Osmonics). Bacterial cells were disrupted and macromolecules extracted with 5 ml of 5% ice cold tricloroacetic acid (TCA) for 5 min. Filters were further processed as previously described (Bell 1993). Disintegrations per min (dpm) were measured with a Beckman Scintillation counter (Beckman LS 6000 IC) after addition of 5 ml Ready Safe (Beckman) scintillation cocktail. We calculated empirical conversion factors for each of the 3 experimental setups (Bottle, Bottle & P, Dialysis — all variants in duplicate) based on the increase of bacterial cell numbers in the <0.8 µm variants and corresponding uptake rates of thymidine. For the calculation of conversion factors, we followed the modified derivative method of Ducklow et al. (1992). Net production rates of the <5 µm variants were determined as the gross productions (measured via radiotracer uptake) minus total grazing rates.

Total and size-fractionated P. Total P (TP) in the <0.8 µm variants was determined spectrophotometrically using the molybdate method after digestion with H_2SO_4 and H_2O_2 (Schmid & Ambühl 1965) from 100 ml subsamples. To achieve a rough separation of the P content of HNF and bacteria in the <5 µm variants, subsamples (100 ml) were filtered at low vacuum (13.3 kPa) onto 1.2 µm pore-size polycarbonate membrane filters (47 mm in diameter, Millipore). TP was estimated for the filters (organisms >1.2 µm corresponding to the 'HNF' fraction) and the filtrates (organisms <1.2 µm corresponding to the 'bacterial' fraction). Therefore, in the <1.2 µm fraction, P was determined as the sum of particulate P and dissolved P (DP). However, values of DP in the raw water were always <1 µg l^{-1} (after filtration through 0.2 µm pore-size filters). In addition, we determined the TP of the unfiltered sample in the <5 µm variants to control the efficiency of the described size differentiation. On 2 sampling dates we took 2 ml of subsamples from the filtrates to account for the potential loss of bacterial cells during filtration (following the staining procedure as described above). At each time point we also analysed samples from the lake to follow the *in situ* TP concentration.

CARD-FISH and biomass of phylogenetic lineages. We applied CARD-FISH (Sekar et al. 2003) with oligonucleotide probes EUB I-III (Daims et al. 1999), ALF968 (Neef 1997), BET42a, CF319a and HGC69a (Amann et al. 1995) to quantify the abundance and biomasses of *Bacteria* (hereafter referred to as EUB), *Alpha-* and *Betaproteobacteria* (ALF and BET, respectively), the *Cytophaga–Flavobacterium–Bacteroides* group (CFB) and *Actinobacteria* (ACT). More details of the hybridization technique are given in Salcher et al. (2007).

Cell dimensions of hybridized bacteria were measured directly at blue excitation (488 nm) with the image analysis system described above. Since CARD-FISH stains cellular proteins, which results in apparently larger cell dimensions than size measurements from DNA staining (DAPI), we applied the following volume-to-C conversion for hybridized cells: $CC_{HYB} = 120 \times MCV_{HYB}^{0.72}$, where MCV_{HYB} is the mean cell volume of hybridized bacteria (μm^3) and CC_{HYB} is the cellular C content of hybridized bacteria (fg C $bacterium^{-1}$). This conversion factor has been previously used to compensate for the larger dimensions of cells stained with Acridine Orange (AO) (Posch et al. 2001). The biomass of bacteria from different phylogenetic lineages (in µg C l^{-1}) was calculated by multiplying CC_{HYB} by their specific abundance (in bacteria l^{-1}).

RESULTS

Bacterial and HNF abundance and biomass

Initial values of bacterial abundance and biomass in the <0.8 µm variants were 3.2×10^6 cells ml^{-1} and 39 µg C l^{-1}, respectively. The release from top-down control caused an increase in bacterial numbers and biomasses in all variants, but to a different extent (Figs. 1 & 2, Table 1). The addition of P resulted in a 2.2- and 3.6-fold rise in numbers and biomasses, respectively. The highest values were found in samples incubated in dialysis tubes. Here, bacterial abundance increased 3.0-fold, and the mean bacterial cell volumes also

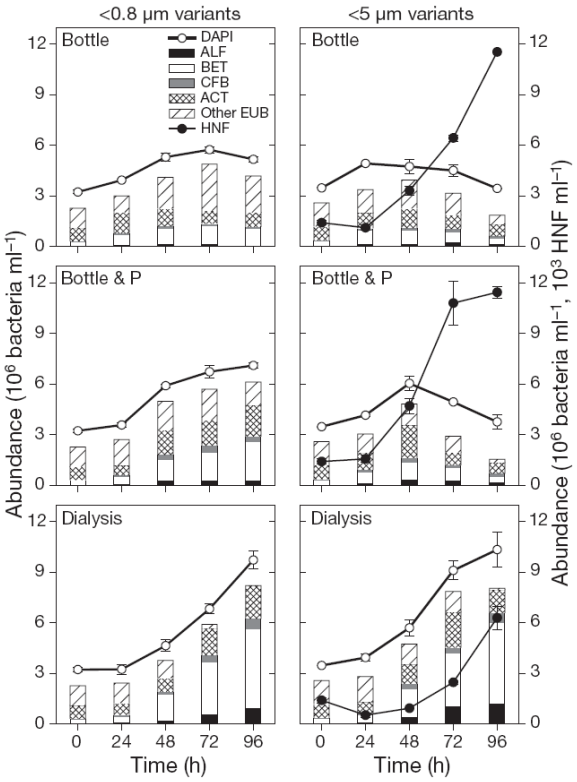

Fig. 1. Development of bacterial (DAPI, ○) and heterotrophic nanoflagellate (HNF) numbers (●) in all experimental variants. Bars show abundances of 4 phylogenetic lineages. Values are means of duplicate setups and whiskers represent the deviation from the arithmetic mean. ALF = *Alphaproteobacteria*, BET = *Betaproteobacteria*, CFB = *Cytophaga–Flavobacterium–Bacteroides* group, ACT = *Actinobacteria*, Other EUB = difference between the sum of specific groups and the abundance of *Bacteria*

increased, which resulted in an 8.9× higher total biomass than at the beginning of the experiment (Fig. 2, Table 1).

As opposed to the <0.8 µm variants, bacterial biomass was negatively affected in all <5 µm variants incubated in bottles where protistan predators were present (Fig. 2). Bacterial abundance and biomass were 3.5×10^6 cells ml^{-1} and 49 µg C l^{-1}, respectively, at the beginning of the experiment. Initial HNF abundance was 1.4×10^6 cells l^{-1}. The addition of P to these variants led to a weak increase in bacterial biomass, but also to a faster increase in HNF numbers (Fig. 1). The highest and relatively stable increase in bacterial biomass (6.0-fold) was again observed in the dialysis tubes (Table 1). In these treatments, the development of protistan grazers was delayed in comparison to the bottle variants (Fig. 1).

Abundance and biomass of bacterial phylogenetic lineages

In the <0.8 µm variants, increases in total numbers could be mainly attributed to BET and ACT. The abundance of ACT increased by a factor of 2.4 after P addition and by a factor of 2.5 after 96 h in the dialysis tubes, but remained stable in unamended bottles. In contrast, numbers of BET were 10 and 20 times higher in these setups, but less than 5 times higher in bottles without P (Fig. 1, Table 1). The specific biomass of BET increased 12 and 28 times after P addition and in the dialysis variant, respectively (Fig. 2, Table 1). The abundance of ALF and CFB also increased in all variants, but their relative contribution to total hybridized cell abundance remained low. However, ALF and CFB reached higher biomass than ACT in the dialysis tubes.

Increasing grazing rates in all <5 µm bottle variants resulted in stagnating or even declining numbers of BET and ACT, whereas CFB and ALF became more numerous (Fig. 1, Table 1). These patterns were also reflected in the development of group-specific bio-

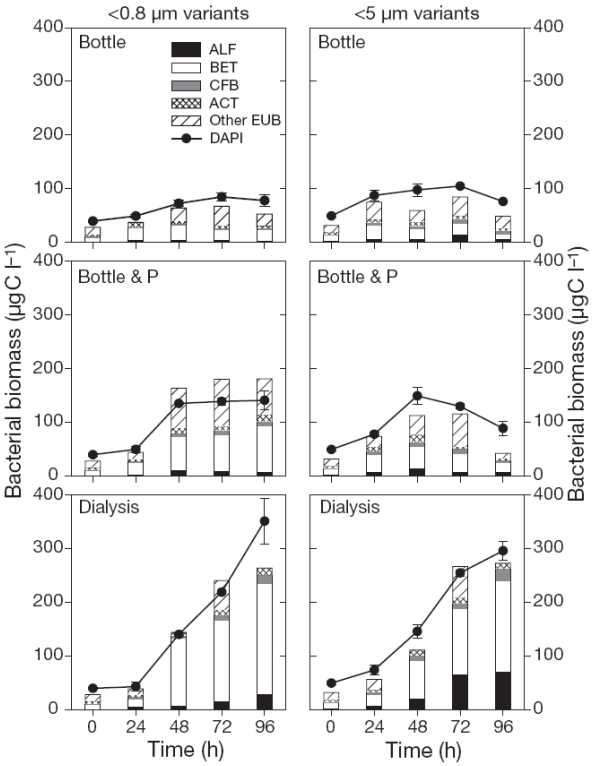

Fig. 2. Development of total bacterial biomass (●) and biomasses of 4 phylogenetic lineages (bars). Values are means of duplicate setups and whiskers represent deviations from the arithmetic mean. For abbreviations see Fig. 1

masses. Since grazing rates in the <5 µm dialysis tubes increased more slowly than in bottles, BET dominated the assemblage in terms of abundance and biomass at the end of the experiment, followed by ALF and CFB (Fig. 2, Table 1).

We compared the BET-to-ACT ratio in terms of bacterial abundance (Fig. 3A,B) and biomass (Fig. 3C,D). ACT was 3 times more abundant than BET at the beginning of the experiment, but BET dominated by a factor of 2 in terms of biomass. The physical separation of bacteria from predators in the <0.8 µm variants shifted numerical dominance from ACT to BET (Fig. 3A). This shift in abundance ratios did not seem to be influenced by P addition, but was very pronounced in the dialysis tubes. Biomass ratios changed even more drastically in the <0.8 µm variants, reflecting a positive effect of P addition and dialysis tube incubations on BET biomass (Fig. 3C). This was due to a pronounced increase in CC_{HYB} in these 2 treatments (Fig. 3E), whereas ACT maintained low C content (Fig. 3G). High grazing rates in the <5 µm bottle variants maintained abundance ratios (Fig. 3B) of the 2 groups as found in the lake and even the biomass ratio (Fig. 3D) remained constant in the unamended bottle. In contrast, biomass ratios were also shifted in favour of BET after P addition and in the <5 µm dialysis tubes.

Table 1. Relative increases (values >1) or decreases (bold values <1) of bacterial parameters (abundance and biomass) after 96 h. Size-fractionated samples (<0.8 and <5 µm variants) were either incubated in bottles (Bottle), in bottles enriched with 15 µg P l^{-1} (Bottle & P) or in dialysis tubes (Dialysis). DAPI = total bacterial parameters determined via DAPI-staining; the following parameters were determined from CARD-FISH preparations: EUB = *Bacteria*, ALF = *Alphaproteobacteria*, BET = *Betaproteobacteria*, CFB = *Cytophaga-Flavobacterium-Bacteroides*, and ACT = *Actinobacteria*

	DAPI	EUB	ALF	BET	CFB	ACT
Bacterial numbers						
<0.8 µm variants						
Bottle	1.6	1.8	2.0	4.2	2.0	1.1
Bottle & P	2.2	2.7	6.8	9.6	6.4	2.4
Dialysis	3.0	3.6	21.2	19.8	11.3	2.5
<5 µm variants						
Bottle	1.0	**0.7**	3.2	1.1	3.0	**0.8**
Bottle & P	1.1	**0.6**	3.7	1.2	3.9	**0.8**
Dialysis	3.0	3.1	24.2	17.5	11.2	1.7
Bacterial biomass						
<0.8 µm variants						
Bottle	2.0	1.9	1.9	2.8	1.3	1.0
Bottle & P	3.6	6.5	4.9	11.6	3.6	3.0
Dialysis	8.9	9.5	18.2	27.9	6.3	2.9
<5 µm variants						
Bottle	1.5	1.6	2.6	**0.8**	3.6	**0.9**
Bottle & P	1.8	1.3	3.1	1.6	2.5	**0.7**
Dialysis	6.0	8.1	28.2	16.3	11.2	1.8

Grazing led to a decrease in the cellular C of BET only in the dialysis treatment (Fig. 3F), whereas the cellular C of ACT increased at the beginning (Fig. 3H).

Bacterial production

If other potential sources of mortality (viral lysis) are neglected, gross production rates in the <0.8 µm variants can be set equivalent to net production rates. In all bottle variants, decreasing production rates after 48 h possibly reflected substrate or nutrient depletion (Fig. 4). Enrichment with P resulted in slightly higher production rates, as also mirrored by elevated bacterial numbers (see Fig. 1). Bacterial production rates showed the most pronounced increase in the <0.8 µm dialysis tubes. Grazing pressure resulted in negative net production already after 48 h (Fig. 4) in the <5 µm bottle variants, but only at the last sampling time point (96 h) in the <5 µm dialysis tubes.

P partitioning

TP concentrations in the <0.8 µm bottle variants were very stable, indicating only small losses due to wall growth of biofilm (Fig. 5). Concomitant with the steep increase in bacterial biomass, we detected a 3-fold increase in P bound in bacteria in the <0.8 µm dialysis variant. In contrast, the TP concentrations of the surrounding lake water remained stable at 6.2 ± 0.4 µg l^{-1} during 4 d (Fig. 5).

The applied separation method of P in all <5 µm variants was very effective, as reflected by linear regression of directly measured TP versus the sum of the 2 P fractions (regression slope = 0.94, r^2 = 0.96, n = 30). Only a negligible overestimation of the real TP concentrations was observed when summing up the 2 size fractions (<10%). Direct counts of bacteria in the <1.2 µm size fractions revealed that on average >95% of bacterial cells of the raw samples passed the filter. In the <5 µm bottle variants, the 2 size fractions accounted for equal amounts of P (Fig. 5), which moreover represented roughly the *in situ* TP concentrations (as determined at time point 0 h). After the enrichment with 15 µg P l^{-1}, approximately half of TP was still bound in the >1.2 µm fraction at 96 h, but 3-fold higher values were reached than in the bottle variants without P enrichment. Interestingly, the accumulation of P by the microbial food web led to an almost equally high increase (2.6×) in particulate P in the <5 µm dialysis tubes. However, less P was found in the largest size fraction than in P-enriched bottles, probably reflecting the slower development of HNF (Fig. 1).

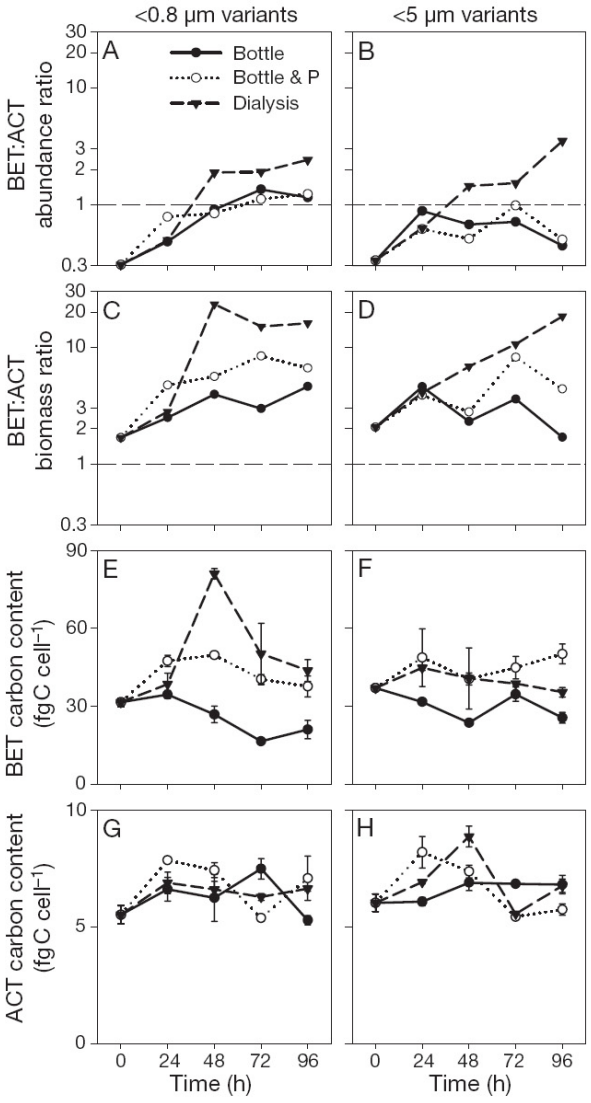

Fig. 3. Ratio between *Beta-* (BET) and *Actinobacteria* (ACT) with respect to their (A,B) abundance and (C,D) biomass over time. Note the logarithmic scale. Cellular C contents of (E,F) BET and (G,H) ACT (note different scale) in all treatments

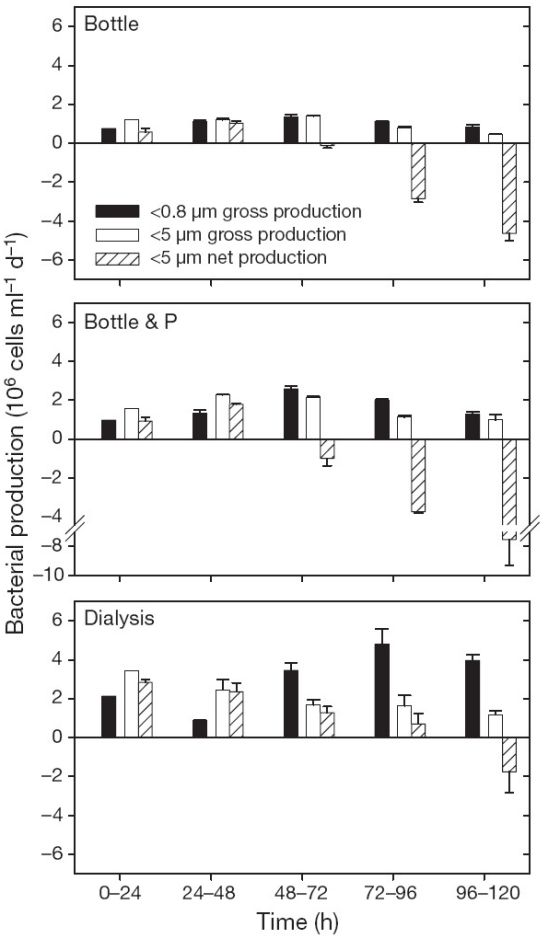

Fig. 4. Bacterial production rates determined via [^{3}H]thymidine uptake rates, which were converted to cell numbers with empirical conversion factors (see text for further explanations). Net production rates for the <5 µm variants were calculated by subtracting total grazing rates from the gross production rates. All values are means of 2 replicate setups; whiskers represent deviations from the arithmetic mean. Intervals at the *x*-axis symbolize periods during which the presented production values are valid

DISCUSSION

Bacterial production potential versus standing stock in Piburger See

In many freshwater lakes, numbers and biomass of heterotrophic bacteria seasonally vary only within a narrow range. However, the determination of bacterial standing stock could be regarded as a 'snapshot,' which does not inform us about the balance of growth and loss processes, and does not take into account substantially higher fluctuations of bacterial productivity. In Piburger See, total bacterial abundance and biomass at the surface layer vary only slightly throughout the year. Sommaruga & Psenner (1995) reported seasonal ranges of bacterial numbers between 1.2×10^6 and 4.7×10^6 cells ml^{-1}. In contrast, leucine uptake rates varied from 0.039 to 1.22 nmol l^{-1} h^{-1}. Even at sampling frequencies of 2 to 3 d, Pernthaler et al. (1996) only found moderate changes in abundance (1.4×10^6 to 3.5×10^6 cells ml^{-1}), whereas thymidine uptake rates varied more than 10-fold (3.7 to 40.2 pmol l^{-1} h^{-1}).

Numerous approaches have been developed to investigate bacterial growth and loss processes in more

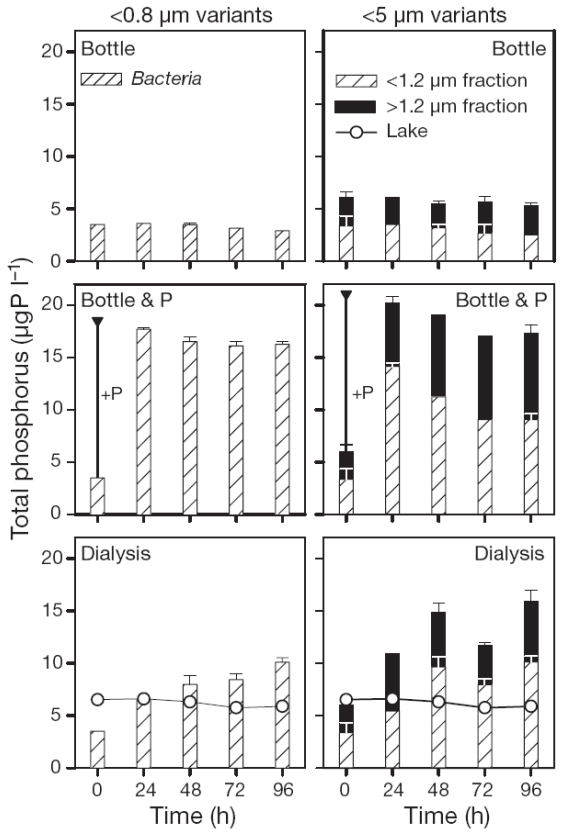

Fig. 5. Total P (TP) concentrations of the bacterial (white bars) and HNF fraction (black bars). For the <5 µm variants, these 2 fractions were separated via filtration over a 1.2 µm pore-size membrane filter. For the Bottle & P variants, the added amount of P (15 µg P l^{-1}) is symbolized by a triangle and a line. TP values of the untreated lake water during the investigation period are shown in the Dialysis panels (○). Values are means of 2 replicate setups; whiskers represent deviations from the arithmetic mean

detail. In the present study, we chose size fractionations of raw water samples to separate bacteria from larger planktonic compartments (Šimek et al. 1999) in combination with nutrient enrichment experiments. Our experimental manipulations resulted in rapid changes of total bacterial abundances that were at least in the range of annual variability. The decoupling of bacteria from larger planktonic components revealed even higher dynamics of P incorporation, production rates and especially of biomasses, reflecting the complexity of processes that lead to the realized levels of bacterial standing stock. These observed changes of bulk parameters could moreover be attributed to the dynamics of distinct phylogenetic bacterial lineages, some of which exhibited disproportional growth or loss compared to the total assemblage (see also Salcher et al. 2007). Specifically, BET showed the highest increase of abundances and biomasses at reduced grazing pressure, while ACT only moderately responded to experimental manipulations.

P enrichment and bacteria as a sink or link of P

As outlined by Vadstein et al. (1993), bacteria can act either as 'sink' or 'link' for different elements in aquatic food webs. Thus, bacteria can contribute in very different ways to the total pool and flux of C and P in ecosystems.

If the development of bacterial parameters in our experiments were regarded only from the unamended bottle variants, the potential production of the microbial assemblage would be significantly underestimated. Addition of P revealed much higher production potential (Fig. 4). Thus, if only bottle incubations with and without P addition were compared, one could conclude that bacterial growth was P limited. However, the incubation of filtrates in dialysis tubes indicated a much more complex situation. A release from grazing pressure illustrated a high growth potential of the total bacterial assemblage and of distinct phylogenetic lineages, if free access to nutrients at ambient concentrations was allowed for (Fig. 2). Therefore, we might need to interpret our addition of P to bottles as a study about the effects of allochthonous (surplus) nutrient addition, rather than as a nutrient limitation experiment.

On the one hand, bacteria showed a high potential to incorporate P after the addition of surplus P in 'bottle systems' (Fig. 5). On the other hand, the bacterial assemblage in the dialysis tubes was capable of accumulating TP at nearly 3-fold higher than ambient levels when released from grazing. These observations indicate that bacteria in Piburger See can act as an effective sink of P at very different concentrations of this nutrient. Few studies have reported the total amount of particulate P within the 'bacterial size fraction' in freshwater lakes. Most of these investigations pointed at a high bacterial contribution (from 30 up to more than 50%) to the pool of TP, typically with high seasonal variability (Rigler 1956, Vadstein et al. 1988, Jürgens & Güde 1990, Rothhaupt & Güde 1992, Vadstein et al. 1993, Elser et al. 1995). In Piburger See, approximately 100% of the TP was found in the size fractions <5 µm, and 50% in the <0.8 µm size fraction during the investigation period (Fig. 5). We conclude that both the bacterial and (presumably) the HNF fraction may act as powerful sinks for P if decoupled from higher trophic levels.

It has been suggested that growth rates of BET may be related to P concentrations (Šimek et al. 2006). Here we provide more direct evidence for a stimulation of

these bacteria by surplus P (Figs. 1 & 2). Moreover BET seemed most effective in collecting P from lake water during incubation in dialysis tubes. This is reflected in the marked increase of P in the bacterial size fraction and the concomitant dominance of bacterial biomass by BET in the <0.8 µm dialysis tubes after 48 h (Figs. 2 & 5). Consequently, our experiments helped to clarify which members within the assemblage profited most strongly from P addition (or were most successful in P acquisition at low ambient levels). By contrast, abundances and biomasses of the total assemblage (all DAPI-stained cells) seemed to be much less affected by grazing or nutrients (Table 1). Thus, one might underestimate the effect of limitation and predation on distinct phylogenetic lineages by studying bacterial bulk parameters only.

Abundances versus biomasses of phylogenetic lineages

Recently, a number of studies have used FISH for analyzing freshwater bacterioplankton. Yet, the majority of these investigations have focused on the quantification of cell numbers but not on biomass. However, a major advantage of the FISH approach is that it simultaneously provides information about both bacterial morphotype and identity, and consequently, about the biomass contributions of different lineages. At present, the quantification of the biomass of hybridized cells is still technically challenging. FISH with fluorescently monolabeled oligonucleotide probes may result in very variable fluorescence intensities of hybridized cells (compared to DNA staining by DAPI), thus complicating the measurements of cell dimensions via standard image analysis procedures. CARD-FISH preparations are characterized by low bleaching and bright signal intensities, but the apparent cell dimensions are typically larger than of DAPI-stained cells. Discrepancies between sizes of cells stained with different fluorochromes are well known, e.g. when using the nucleic acid stains DAPI and AO (Sieracki & Viles 1992). For the correct quantification of biomass (in µg C l^{-1}), such differences can be compensated for by the use of adequate, stain-specific volume-to-C conversion factors (Posch et al. 2001). Unfortunately, to date, there is no rigorous comparison of cell sizes determined by CARD-FISH and other fluorochromes. Therefore, we applied a volume-to-C conversion factor, which was originally described for AO-stained cells, to calculate biomasses of hybridized cells. Considering the staining properties of AO (it interacts with DNA and RNA), this seems to be a good approximation. Moreover, the biomasses of all hybridized cells (stained by probe EUB I–III) seemed to match the biomasses determined from DAPI-stained preparations reasonably well at time points when the vast majority of DAPI-stained cells could be hybridized (see our Fig. 2 and Salcher et al. 2007). In any case, the biomass ratios (Fig. 3) and contributions of different lineages to total biomass of hybridized cells (Fig. 6) are unaffected by conversion factors.

The determination of the abundances and biomasses of distinct phylogenetic lineages revealed complementary aspects of the studied microbial assemblage (Fig. 6). Although ACT numerically dominated the assemblage *in situ*, their biomass was only half of BET. This discrepancy was even more pronounced in all

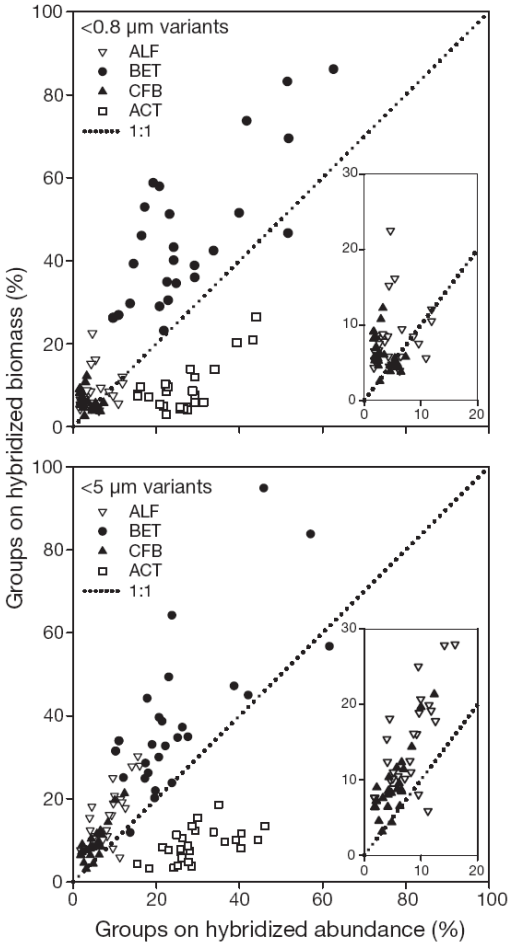

Fig. 6. Abundance and biomass contributions of 4 phylogenetic lineages to total hybridized *Bacteria* (determined via hybridization with probe EUB I–III). All data from the bottle variants (with and without P addition) and the dialysis variants were pooled for the <0.8 µm and the <5 µm setup, respectively. Note the 2 inserts highlighting development of *Alphaproteobacteria* (ALF) and *Cytophaga–Flavobacterium– Bacteroides* (CFB). BET = *Betaproteobacteria*, ACT = *Actinobacteria*

variants where HNF were removed via filtration (Fig. 3A,C). This may point to the importance of size-selective protistan grazing in affecting biomass ratios of different phylogenetic groups. This aspect is also likely to influence the competition between bacterial lineages (Salcher et al. 2007).

At present, it is still unclear if abundance or biomass represents a more appropriate link to the activity patterns of distinct phylogenetic lineages (Yokokawa et al. 2004). While there is evidence for a correlation between relative abundance and the percentage of ^{3}[H]leucine active cells for distinct lineages in freshwater systems (Zhang et al. 2006), this relationship may change dramatically due to substrate availability and quality (Pérez & Sommaruga 2006). Therefore, it might be necessary to synoptically study the contribution of different bacterial lineages to the activity, abundance and biomass dynamics of the total microbial assemblage in the context of top-down control and nutrient limitation. The methodological tools for such investigations have become available, namely quantification of lineage-specific biomasses via image analysis procedures, analysis of food vacuole contents of bacterivores (Jezbera et al. 2006), and specific uptake preferences via microautoradiography (Zhang et al. 2006). Thus, the combination of these approaches might substantially refine our view on C and P fluxes within microbial food webs.

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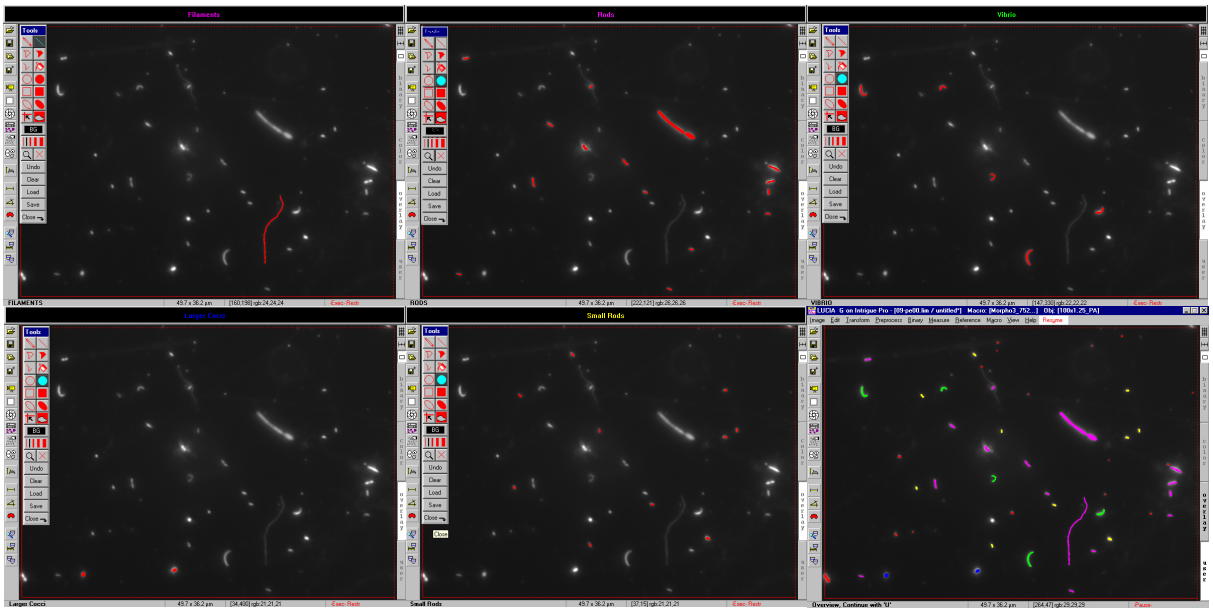

manuscript IV

Morphotype specific contributions of hybridized cells to total bacterioplankton abundance and biomass as studied by image analysis. Positive relation of cell size to hybridization efficiency?

Posch T, Franzoi J, Prader M & Salcher MM

(submitted)

Morphotype specific contributions of hybridized cells to total bacterioplankton abundance and biomass as studied by image analysis. Positive relation of cell size to hybridization efficiency?

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Abstract

We present an image analysis routine to determine the contribution of distinct morphotypes to the total abundance and biomass of freshwater bacterioplankton and to the fraction of cells detected by fluorescence *in situ* hybridization (FISH). The method was tested on bacterial assemblages from a prealpine lake (Piburger See, Austria) at characteristic time points during the limnological year. Although on average <50% of DAPI (4',6'-diamidino-2-phenylindole) stained particles were hybridized with the oligonucleotide probe EUB I-III, we detected most of total biomass determined from DAPI-staining (up to 89%). The assemblage was numerically dominated by minute cocci and rods <0.6 µm (mean cell volume = 0.024 μm^3). Only a minor part of these morphotypes could be hybridized (15% and 40%, respectively). In contrast, larger rods (0.087 μm^3), cocci (0.155 μm^3) and vibrio-shaped cells (0.073 μm^3) showed much higher probabilities to be detected by FISH. These morphotypes *per se* formed the highest contribution to total biomass which explained the high detection efficiency of biomass with FISH. Further we determined morphotype distributions within three distinct phylogenetic lineages. *Actinobacteria* were predominately small rods and cocci, whereas bacteria from the *Cytophaga-Bacteroides-Flavobacterium* group formed mainly large rods and filaments. *Betaproteobacteria* showed the highest morphological variability. Our analysis illustrates that most bacterial carbon biomass can be analyzed, even at low numerical detection rates by FISH. Thus, the here presented approach could help to elucidate the relationship between geno- and morphotype in aquatic microbial food webs and to study carbon flow through different bacterial taxa in more detail.

Introduction

Planktonic freshwater bacteria differ in their phenotypes, genotypes and their physiological properties. It is a challenging task to develop methods at the single cell level that simultaneously inform on more than one of these bacterial characteristics. The combination of fluorescence *in situ* hybridization (FISH) and DNA-staining with the fluorochrome DAPI (4', 6'-diamidino-2-phenylindole) has to be regarded as one highlight in this context (11). A further improvement of FISH via combination with microautoradiography techniques indeed informs about the taxonomic affiliation, the phenotype and activity patterns (16). Although FISH allows for the simultaneous analysis of the morpho- and genotype, most studies have only focused on the numerical importance of distinct freshwater bacterial lineages, i.e., on cell numbers or on percentages of the total assemblage (4, 37), rather than on biomasses (5, 24, 38). Many investigations using the oligonucleotide probes EUB338 or EUB I-III for the detection of *Bacteria* (7) share one characteristic observation: Detection rates of bacteria, hybridized with these probes, are far below 100% of DAPI-positive particles. Bouvier & del Giorgio (4) reported an average value of 50% for freshwater systems. Even a further improvement of FISH via catalyzed reporter deposition (CARD-FISH) did not result in much higher detection rates within freshwater assemblages of certain lakes (33). This either points to methodological problems of the FISH method itself (4), or to an imprecise reference, namely an overestimation of total bacterial abundance. DAPI as the typical reference dye for FISH may also stain viruses (2) or even detrital particles, thus potentially leading to imprecise bacterial numbers.

In this study, we highlight that hybridization with probe EUB I-III allows detection of a large fraction of freshwater bacterioplankton biomass, even when the hybridization rates are relatively low. We present an image analysis procedure to discriminate between different bacterial morphotypes and to assess their contribution to total bacterial numbers and biomasses. In a second step, we analyzed which of these morphotypes were detected by FISH, and evaluated their contribution to the total assemblage. First tests are presented to link the information on morphotypes with a taxonomic affiliation by applying three specific oligonucleotide probes.

Materials and Methods

Study site and sampling

Piburger See, a dimictic holomictic lake, was strongly affected by anthropogenic induced eutrophication during the 1960ies and 1970ies. Since the installation of an Olszweski tube for removal of hypolimnetic water in 1970, an oligo-mesotrophic status was reached again only after 30 years (35). In May 2005, shortly after the phytoplankton spring bloom, samples were taken with a 5 l Schindler sampler at 3 m intervals from 0 to 24 m depth (maximum depth = 24.6 m). In June 2005, October 2005, and January 2006 we took samples in four selected depths (3, 9, 18, and 24 m) to follow the seasonal patterns of hybridized bacteria (i.e., summer, autumnal and winter stratification). Total bacterial abundance was determined also in 3 m intervals for the whole depth profile. Oxygen concentrations (Winkler-method) and chlorophyll *a* concentrations (photometry after extraction) were determined according to standard techniques.

Total abundance of DAPI-positive particles

40 ml samples were fixed with 0.2 µm pre-filtered formaldehyde (3% final conc.) and processed within one week to avoid cell losses (36). Subsamples (2 – 3 ml) were stained with DAPI (4 µg ml^{-1} final conc., 7 min) (27) and filtered on black polycarbonate filters (0.22 µm pore size, Osmonics). We used a Zeiss Axioplan Microscope with an optovar for additional magnification (1.25x, 1.6x, 2.0x and 2.5x) and a filter set Zeiss 01 (BP 365, FT 395, LP 397) for UV-excitation. We counted 700 to 1000 DAPI-stained particles per subsample at a magnification of 2000x.

CARD-FISH

Samples of 10 ml were fixed with paraformaldehyde (2% final conc., adjusted to pH 7.4), filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2 µm pore size, 47 mm diameter), and stored at -20° C until further processing. CARD-FISH was carried out as previously described by Sekar et al. (33) using the following HRP-labeled probes: EUB I-III for *Bacteria* (7), BET42a for *Betaproteobacteria* (19), CF319a for members of the *Cytophaga-Flavobacterium-Bacteroides* group (20), and HGC69a for *Actinobacteria* (29). Signal-amplification was carried out with ALEXA 488 labeled tyramides. Hybridized cells were analyzed at blue excitation (filter set Zeiss 09, BP 450-490, FT 510, LP 515).

Image analysis procedure (Fig. 1 & 2)

We used the image analysis software LUCIA G/F (Laboratory Imaging, Prague) for the determination of organism's dimensions and morphotypes (28). The system has a user-friendly and simple macro-language, which allowed for an extensive automation of the workflow (Fig. 1). Pictures were taken with a Vosskühler CCD camera (12 bit, resolution = 976×756 pixel) at a magnification of 125x resulting in a pixel length of 0.055 µm pixel^{-1}. LUCIA G/F works with 8 bit images per channel, corresponding to 256 possible gray values per pixel (0 = black, 255 = white). One image can be composed of up to three gray channels (1-3), resulting in a pseudo color RGB image. We took two images from the same field of view, one at UV-excitation for the detection of all DAPI-positive cells (Channel 1) and a second at blue excitation for the detection of hybridized bacteria within the same microscopic field (Channel 2). First, we detected all cells of the two channels resulting in two binary images (see details below). Afterwards, all morphotypes of channel 1 (DAPI-positive cells) were measured. Binary images of both channels 1 & 2 were combined by an object-based 'AND' function, thus selecting those DAPI-positive cells in the binary image of channel 1, which had a counterpart also in the binary image of channel 2 (hybridized bacteria). Thereafter, cell sizes and morphometric parameters were measured from channel 1 again (Fig. 1). By this procedure we estimated also the percentage of hybridized in total DAPI-positive bacteria.

Morphotype detection of total and hybridized bacteria (Fig. 1)

We applied a 'Mexican Hat'-type edge detection filter (kernel size = 5×5 pixel) which equalizes gray values of all pixels within an object to a value of 255 (24). A threshold value of 255 was set by default for the generation of the binary image. Although the filter works excellently for the detection of even very dim cells it slightly overestimates the actual cell dimensions. To reduce the size of objects in the binary image we applied morphological "eroding-", "opening-" and "smoothing-" procedures (28). A morphological "cleaning" of the binary image removed all objects consisting of less than 5 pixel. Prior to measurements the original gray image was overlaid with the red colored binary image for controlling the effectiveness of the edge detection process and interactively deleting objects other than bacteria (flagellates or detrital particles). This workflow was done for both image channels 1 & 2.

To distinguish bacterial morphotypes we applied a sequence of parameter restrictions which allowed the stepwise discrimination of the following morphotypes (Fig. 1 & 2A-D): filaments, large rods, vibrio-shaped cells, large cocci, small rods, small cocci, and rare irregular morphotypes. Before cells of a distinct morphotype-class were measured, we overlaid the original gray image with a binary image presenting solely cells from this class. By this we could interactively delete misclassified objects. The manually removed objects were maintained in the binary image of the next detected class of morphotypes and, therefore, were not lost for the analysis (Fig. 1 & 2). Prior to the actual evaluation, we tested different sequences and combinations of discrimination parameters to obtain the fewest misidentifications. The analysis was started with the detection of filaments defined as cells with a *Length*-parameter (see Table 1) larger than 5 µm. In a next step larger rod-shaped bacteria were detected. We noticed that DAPI-stained rods of Piburger See had a cell width of 0.26 to 0.33 µm. The length distribution of all rod shaped bacteria revealed a maximum for small rods at 0.55 µm (Fig. 2D).

By applying a restriction parameter of *Elongation* >2.3 (Fig. 2A) we obtained large rods with a minimum cell length of approximately 0.65 µm (Fig. 2C). In addition we set a second restriction of C*ircularity* <0.85 to avoid the detection of large coccoid cells. For the selection of the third morphotype-class, namely vibrio-shaped cells, we chose restrictions of *Elongation* between 1.1 to 5 in combination with *Circularity* <0.7 (Fig. 2B). The shape of vibrios was characterized by very low circularity values (Fig. 2B) as these cells have a large perimeter in comparison to a small area. Most vibrio shaped cells had *Elongation* values <2.3, however we set the range of *Elongation* relatively wide (between 1.1 to 5) as some rare vibrio-shaped cells showed values up to 3 (Fig. 2A) The detection of larger coccoid cells (>0.6 µm) was done by a combination of three restrictions, i.e. *Elongation* <2.5, *Circularity* >0.5 to <1 and *MaxFeret* >0.6 (Fig. 2C), whereby these combined classifiers allowed for an efficient exclusion of rare irregular morphotypes. In the next step small rods (<0.6 µm) were analyzed by the restriction of *Circularity* <0.95, small cocci (<0.6 µm) had a *Circularity* >0.95. Finally we reset all discrimination parameters to look for rare morphotypes, which were occasionally represented by prosthecate cells.

In total, 800-1200 DAPI-positive cells were measured per sample (equivalent to 20-40 images), resulting in the detection of 300-600 hybridized cells in channel 2. The following parameters were determined for each cell: *Area, Perimeter, Length, Width, Maximum Feret dimension, Minimum Feret dimension, Elongation, Circularity* and *Class* (definitions are given in Table 1, see also Fig. 1). For the calculation of bacterial cell volumes we selected the adequate length and width. For cells were *Length* (calculated from area and perimeter, Tab.1) was larger than *Maximum Feret*, we selected the *Length* and the corresponding *Width*. In all other cases the *Feret* dimensions were used, whereby this step was done automatically with an Excel-macro. Mean bacterial cell volumes of all morphotypes were calculated by modeling cells as cylinders with two spherical ends (Table 1). This formula can also be applied for spherical cells, as the term “Length – Width” approaches zero for coccoid cells. Bacterial cell volumes were converted to cellular carbon contents applying the allometric formula by Loferer-Krößbacher et al. (18), assuming that carbon corresponds to 50% of the dry weight (Table 1). Bacterial biomass (in µg C l^{-1}) was calculated by multiplying the cellular carbon content by bacterial abundance.

Statistical analyses

Regression analyses were performed with the software SigmaPlot 8.02 (SPSS Inc.). Differences in the relative abundances of the individual morphotypes within individual phylogenetic groups were established by 2-way ANOVA using a nested factorial design (morphotypes nested within phylogenetic groups). In order to account for the different data distributions the relative abundances of morphotypes were arcsine transformed prior to analysis. After the establishment of significant interactions between the two factors, differences between the fractions of morphotypes within each phylogenetic group were separately analyzed by 1-way ANOVAs and post-hoc pairwise comparisons (Scheffé method). Analyses were performed using the software SPSS (SPSS, Inc., Chicago, Ill., USA).

Results

Samples taken in May 2005 characterized the situation shortly after the phytoplankton spring bloom, which was formed by algae of the classes *Cryptophyceae* and *Chrysophyceae*. The lake was already thermally stratified and oxygen was not yet depleted above ground (Fig. 3). Maximal numbers of heterotrophic bacteria developed below the chlorophyll *a* maxima and above ground (Fig. 3). We observed higher detection efficiencies of bacterial biomass rather than abundance with the oligonucleotide probe EUB I-III (Fig. 3). Pooled data of the profile from May 2005 indicated the following morphotype composition (Fig. 4): small cocci (50% of total cells) > large rods (18%) > small rods (17%) > large cocci (10%) > vibrio-shaped bacteria (5%). Although small cocci numerically dominated, they represented a low contribution to total bacterial biomass (20%). Large rods (28%) and large cocci (31%) formed most of the total biomass (Fig. 4). The detection efficiency with probe EUB I-III was 30-50% (mean = 36%) of total bacterial abundance (Fig. 4 & 5). The majority of large morphotypes were hybridized, whereas only 15% of small cocci were detected (Fig. 4 & 5B). The highest fractions of hybridized cells were found within large cocci and vibrio-shaped cells (Fig. 5B). Due to the high contribution of larger cells types, up to 83% of the total biomass could be detected via hybridization (mean = 66%). While larger morphotypes generally had higher detection probabilities, this did not hold true for small rods and vibrio-shaped bacteria (Fig. 5B). However, there was a significantly positive correlation between bacterial cell length class and the fraction of therein hybridized bacteria. The best fitting regression curve (Fig. 5C) was found by a sigmoid model (r^2 = 0.84). Hybridized cells (y-value) within a size class (x-value) could be calculated by the equation: $y = 82.6 / (1+e^{(-(x - 0.555) / 0.163)})$.

In June and October 2005 chlorophyll *a* peaks reflected the occurrence of the diatom *Asterionella* sp. The zone of hypolimnetic oxygen depletion reached up to 15 m in October 2005 (Fig. 6). Although depth profiles of total bacterial abundance and biomass changed during the seasonality of the lake (Fig. 6), the detection efficiencies of different morphotypes with probe EUB I-III confirmed our observations from May 2005. In addition, filaments had developed in some depths. On average 50% (range: 38-64%) of DAPI-positive cells could be hybridized, corresponding to 77% (range 58-88%) of total biomass (Fig. 6). Total abundance increased above the sediment on all sampling dates. Highest values were reached during the autumnal thermal stratification, indicating a prominent assemblage of potentially microaerophilic or anaerobic bacteria. In January 2006 the bacterial assemblage was homogeneously distributed down to 21 m, mirroring the period after holomixis (Fig. 6).

For the last three sampling dates we determined the morphological composition of 3 phylogenetic lineages (Fig. 7, Table 2). *Actinobacteria* formed small morphotypes, mainly small rods (volume = 0.018 μm^3) and cocci (0.028 μm^3), and there were some larger rods with minimal cell widths and consequently low cell volumes (0.024 μm^3). We never observed filamentous morphologies of this lineage. In contrast, all filaments belonged to the *Cytophaga-Flavobacterium-Bacteroides* group, which appeared also as large rods and cocci (Fig. 7, Table 2). A clear correspondence between morphology and taxonomic affiliation could not be observed for *Betaproteobacteria*. The morphological variability in this group was high, closely mirroring the size distribution of *Bacteria* (Table 2). Due to their varying

morphological composition and cell sizes, the contribution of specific lineages to total biomass differed from their numerical importance (Table 3). Although *Actinobacteria* were the most abundant group (17% - 23% of DAPI-positive particles), their percentage of total biomass was minimal (5% - 11%). The opposite was observed for *Betaproteobacteria* and the *Cytophaga-Flavobacterium-Bacteroides* group (Table 3).

Discussion

Methodological remarks

First studies using FISH for analyzing freshwater systems were mainly based on Cy3-labeled oligonucleotide probes, see Table 1 in (4). The fluorescence properties of hybridized bacteria were hampered by the low intensities (especially of small morphologies) and the fast fading of fluorescence signals. The introduction of CARD-FISH solved some of these drawbacks, allowing the detection of even minute cells and yielding bright fluorescence signals of hybridized bacteria. Nevertheless, also CARD-FISH can lead to hybridization rates in freshwater systems which are below 100% of total cells determined via DAPI staining (33). However, it is still not clear if DAPI stained preparations should be regarded as the gold standard to evaluate hybridization rates with the probe EUB I-III. This 'DNA-specific' dye may lead to unspecific bindings and many other objects than bacteria can be stained, i.e. viruses, free DNA adsorbed to particles and submicrometer detrital particles (2, 40**)**. Nevertheless, it would be too simple to regard all small particles as non-bacterial morphologies. In Piburger See 15 % of all minute cocci and 40% of small rods could be hybridized with probe EUB I-III (Fig. 5). As a last explanation of low hybridization rates, an insufficient coverage of the domain *Bacteria* by the oligonucleotide probe EUB I-III could be envisaged, as only approximately 90% of sequence types in the database of the Ribosomal Database Project (http://rdp.cme.msu.edu/) are targeted by the probe. There is still need for future studies elucidating the characteristics of non-hybridized particles in freshwater systems.

In general, hybridization rates with the probes EUB338 or EUB I-III are low (<60%) for bacteria of various freshwater lakes (4, 37). However, there are also opposing reports. Higher hybridization rates were found for manipulation experiments, e.g. size fractionations or nutrient enrichments. In this context, increased hybridization rates could be often attributed to the disproportional growth of distinct lineages with large cell sizes, e.g. *Betaproteobacteria* (30). Some investigators reported on high EUB-detection rates also for un-manipulated lake samples, surprisingly even for the bacterioplankton of the ultra-oligotrophic high mountain lake Gossenköllesee (1, 23, 24, 25). However, the total bacterial assemblage of that lake with an abundance of 10^5 cells ml^{-1} was clearly dominated by larger morphologies with cell volumes of 0.06-0.09 μm^3 (24). Own observations in Gossenköllesee revealed a dominance of large rods, vibrio-shaped cells and filaments, whereas small cocci and rods together represented only 20% of total DAPI-positive bacteria (unpubl. data.). Consequently, a positive correlation between the size of a bacterium and its probability to be hybridized could be suggested (24). Such a relationship seems to be reasonable for monolabeled oligonucleotide probes. The cellular ribosome content is probably linked to cell size and therefore hybridization rates would increase with cell dimensions. When applying CARD-FISH, this correlation should be less clear, as even few bound

probe molecules would result in strong signal amplification (32). However, in this study, we found indications for a positive relationship between cell size and hybridization success, at least for the hybridization with probe EUB I-III (Fig. 5C). Concerning different morphotypes we found exceptions, e.g. medium-sized vibrio-shaped bacteria showed much higher detection probabilities as would have been expected (Fig. 5B). Also small rods were more efficiently detected than small cocci, although these two morphotypes had equivalent cell volumes (Fig. 5A). This could be an indication that the latter morphotype may harbor objects of non-bacterial origin. The relation between cell size and hybridization probability becomes even more complex when applying specific oligonucleotide probes. The hybridization of *Actinobacteria* seemed to be very effective (Table 3), although all detected cells had very small cell dimensions.

Significance of morphotypes

Already in the year 1933, A.T. Henrici (see plate 1 in (10)) pointed at the huge variety of bacterial morphotypes in lakes, criticizing that the isolation of freshwater bacteria on culture plates does not mirror the morphological variability *in situ*. As the quantification of bacterioplankton is mainly based on direct counting techniques, an extensive knowledge of the ecological significance of bacterial morphotypes would be expected. Moreover, several authors have presented sophisticated image analysis routines for an automated detection of bacterial morphotypes from various ecosystems (3, 8, 17). Surprisingly, to date only few studies reported on the morphotype composition of freshwater bacterioplankton (9, 31) and on the seasonal succession of morphotypes (13, 15). Some conspicuous morphotypes (filaments, prosthecate bacteria) have been studied in more detail; e.g. in the context of predation pressure by bacterivorous protists (14). Image analysis systems even allowed to link information about distinct larger morphotypes with cell specific physiological activities, like phosphatase activity (21), uptake of radiolabelled substrates (6, 22) or respiratory activity (28). In contrast, the functional role of less conspicuous morphotypes, e.g. small cocci, rods and vibrio-shaped bacteria, is still poorly investigated, although these forms can dominate freshwater bacterioplankton. Striking ideas about the ecological and evolutionary significance of bacterial cell morphology are explored in the review of K.D. Young (39). The author lists multiple examples of how bacterial morphology can be linked to nutrient access, the bacterial cell cycle, attachment, motility, and predation. Currently, most observations are still based on lab experiments with isolated bacteria. Single or multiple nutrient limitations can strongly affect the morphological variability of single strains, e.g. of *Vibrio* sp. (12) or *Caulobacter* sp. (26). Regarding field studies, it seems that our knowledge is still restricted how grazing pressure and especially nutrient limitation will form the *in situ* composition of less conspicuous bacterial morphotypes and their activities (34). In this context the high surface-to-volume ratios of small thin rods and vibrio-shaped cells (39) could be regarded as one adaptation-strategy to low nutrient concentrations in freshwater systems.

Morphotypes and biomasses of specific lineages

In situ hybridization techniques allow for linking morphotypic with genotypic information. In Piburger See, some phylogenetic lineages formed specific morphotypes (Fig. 7), e.g. *Actinobacteria*

were predominately small rods and vibrios. Such clear patterns were not observed for *Betaproteobacteria*, which exhibited a high morphological variability, possibly reflecting a high phylogenetic or growth status diversity within this group. Moreover, it happened that a single phylogenetic lineage, although present at low abundance, can dominate the total bacterial biomass and *vice versa* (Table 3). Thus, the measurements of cell size and shape of bacteria from specific lineages will be a prerequisite to obtain a more detailed picture of biomasses, production and carbon fluxes in microbial food webs (38, 41) and to link morphotypes to ecophysiological adaptations.

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Figure legends

Figure 1: Flow diagram of the image analysis routine for quantifying 7 different morphotypes of the total bacterial assemblage (DAPI-image, channel 1). The contribution of hybridized cells (FISH-image, channel 2) can be assessed by working with double layer images of the same microscopic field. Gray images of five morphotypes and the corresponding binary images are shown. See text and Table 1 for further explanations.

Figure 2: Examples for the use of parameter restrictions allowing for the stepwise discrimination of bacterial morphotypes (A-C), for details see text. Gray shaded boxes indicate morphotypes already excluded from the stepwise detection of morphotypes. Large rods were defined as cells with an *Elongation* >2.3 (Fig. 2A) and a *Circularity* < 0.85. Afterwards vibrio shaped cells were detected by the restriction Circularity <0.7 (Fig. 2B). Thereafter, large cocci were defined by *Maximum Feret* >0.6 µm (Fig. 2C). The bars show the 25^{th}, 50^{th}, and 75^{th} percentiles of all data, whiskers stand for the 10^{th} and 90^{th} percentiles, and black circles indicate the 5^{th} and 95^{th} percentiles (n = 1200 measured bacteria). Diamonds symbolize the arithmetic means. The size distribution of all rod shaped cells indicated a peak of small rods (average length = 0.55 µm) and a wide size range of larger rods (Fig. 2D).

Figure 3: Sample profiles taken from Piburger See (Austria) in May 2005 shortly after the phytoplankton spring bloom. (A) Profiles of temperature, oxygen, chlorophyll *a*. Abundance (B) and biomass (C) of all DAPI-positive particles (Total) and of cells hybridized with the oligonucleotide probe EUB I-III (EUB).

Figure 4: Top figures: Contribution of five different bacterial morphotypes to the total number and biomass of DAPI-positive particles (= 100%) in Piburger See (Austria, May 2005). Lower figures: Percentages of DAPI-positive morphotypes, also hybridized with the oligonucleotide probe EUB I-III, to the total abundance and biomass. The width of each column resembles the total EUB detection rate (per each depth: n=800-1200 DAPI positive cells corresponding to 300-600 hybridized cells).

Figure 5: (A): Mean cell volumes of EUB I-III hybridized morphotypes. (B): Detection frequency of different bacterial morphotypes by hybridization with the oligonucleotide probe EUB I-III. The bars show the 25^{th}, 50^{th}, and 75^{th} percentiles of all data and whiskers stand for the 10^{th} and 90^{th} percentiles. (C): Relationship between bacterial cell length class and the fraction of therein hybridized bacteria. The sigmoid regression ($r^2 = 0.84$) determines the hybridized cells (y-value) within a size class (x-value): $y = 82.6 / (1+e^{(-(x - 0.555) / 0.163)})$.

Figure 6: Left: Profiles of temperature, oxygen, chlorophyll *a* at summer (June 05) and autumnal stratification (October 2005), and after mixis (January 2006) in Piburger See (Austria). Contribution of six different bacterial morphotypes hybridized with probe EUB I-III to the total number (middle figures) and biomass (right figures) of DAPI-positive particles.

Figure 7: Morphotype frequency patterns of four phylogenetic lineages in Piburger See (Austria). Bacteria were hybridized with the following oligonucleotide probes: EUB I-III for *Bacteria*, BET42a for *Betaproteobacteria*, HGC69a for *Actinobacteria* and CF319a for the *Cytophaga-Flavobacterium-Bacteroides* group. Values are pooled from three sampling dates and four

sampling depths. The bars show the 25th, 50th, and 75th percentiles of all data, whiskers stand for the 10th and 90th percentiles, and black circles indicate the 5th and 95th percentiles.

Table 1: Definition of cell specific parameters determined with the image analysis software LUCIA G/F (Laboratory imaging, Prague), (Pr = Projection).

Parameter	**Method of determination**	**Comment**
Area (μm^2)	∑(pixels within an object) × (pixel area)	Useful for calculation of cell dimensions.
Perimeter (µm)	$0.25 \times \pi \times (Pr_0 + Pr_{45} + Pr_{90} + Pr_{135})$	Useful for calculation of cell dimensions.
Length (µm)	$0.25 \times (Perimeter + \lvert Perimeter^2 - 16 \times Area \rvert^{0.5})$	Reliable length-estimation for curved cells and filaments.
Width (µm)	*Area / Length*	Reliable width-estimation for curved cells and filaments.
Maximum Feret (µm)	Maximum value between tangents circulating at angle α = 0, 10, 20,….180° around the object.	Reliable length-estimation of straight rods and coocoid cells.
Minimum Feret (µm)	Minimum value between tangents circulating at angle α = 0, 10, 20,….180° around the object.	Reliable width-estimation of straight rods and coocoid cells.
Elongation	*Maximum Feret / Minimum Feret*	Allows the detection of rods.
Circularity	$4 \times \pi \times Area \times Perimeter^{-2}$	Value = 1 for circles, <1 for rods. Vibrios have very low values.
Morphotype Class	Assigned value between 1 and 7 depending on morphological characteristics.	Characterizes the morphotype (e.g. filaments = class 1, large rods = class 2,….).
Volume (μm^3)	$(4/3 \times 0.125 \times Width^3 \times \pi) +$ $(Length - Width) \times (0.25 \times Width^2 \times \pi)$	Cells are regarded as a cylinder with two spherical ends.
Carbon content (fg C $cell^{-1}$)	$218 * Volume^{0.86}$	Allometric formula regards the higher carbon to volume ratio of small cells (reference (18)).

Table 2: Differences in the relative abundances of morphotypes within individual phylogenetic groups as established by 2-way ANOVA and post-hoc pairwise comparisons (Scheffé method). Identical numbers indicate groups that are statistically indistinguishable. -1 = significantly lower percentage than group(s) 0; +1 = significantly higher percentage than group(s) 0.

	EUB I-III	**BET42a**	**HGC69a**	**CF319a**
Small cocci	+1	0	+1	0
Small rods	0	0	+1	-1
Vibrio shaped	0	0	0	-1
Large rods	0	0	-1	+1
Large cocci	0	0	-1	+1
Filaments	0	0	0	+1

Table 3: Contribution of bacteria hybridized with different oligonucleotide probes (for probe abbreviations see text) to total abundance of all DAPI positive bacteria. Biomass (mean ± standard deviation) of all DAPI positive bacteria and of hybridized bacteria. Contribution of hybridized bacteria to total bacterial biomass and to the biomass of *Bacteria* (hybridized with probe EUB I-III). Data were pooled for three sampling dates (June and October 2005, January 2006) for the aerobic depth zones (n=8) and the anaerobic depth zone (n=4).

	ALL DAPI	**EUB I-III**	**BET42a**	**HGC69a**	**CF319a**	**Sum of probes**
% of total abundance						
Aerobic zone	100	48	10	23	4	37
Anaerobic zone	100	61	25	17	9	51
Biomass (µg C l^{-1})						
Aerobic zone	35 ± 9	26 ± 7	6 ± 3	4 ± 1	5 ± 1	15 ± 5
Anaerobic zone	88 ± 60	73 ± 49	24 ± 9	4 ± 0	31 ± 12	59 ± 21
% of total biomass						
Aerobic zone	100	74	18	11	15	44
Anaerobic zone	100	83	27	5	35	67
% of EUB I-III biomass						
Aerobic zone	-	100	24	16	20	60
Anaerobic zone	-	100	33	6	42	81

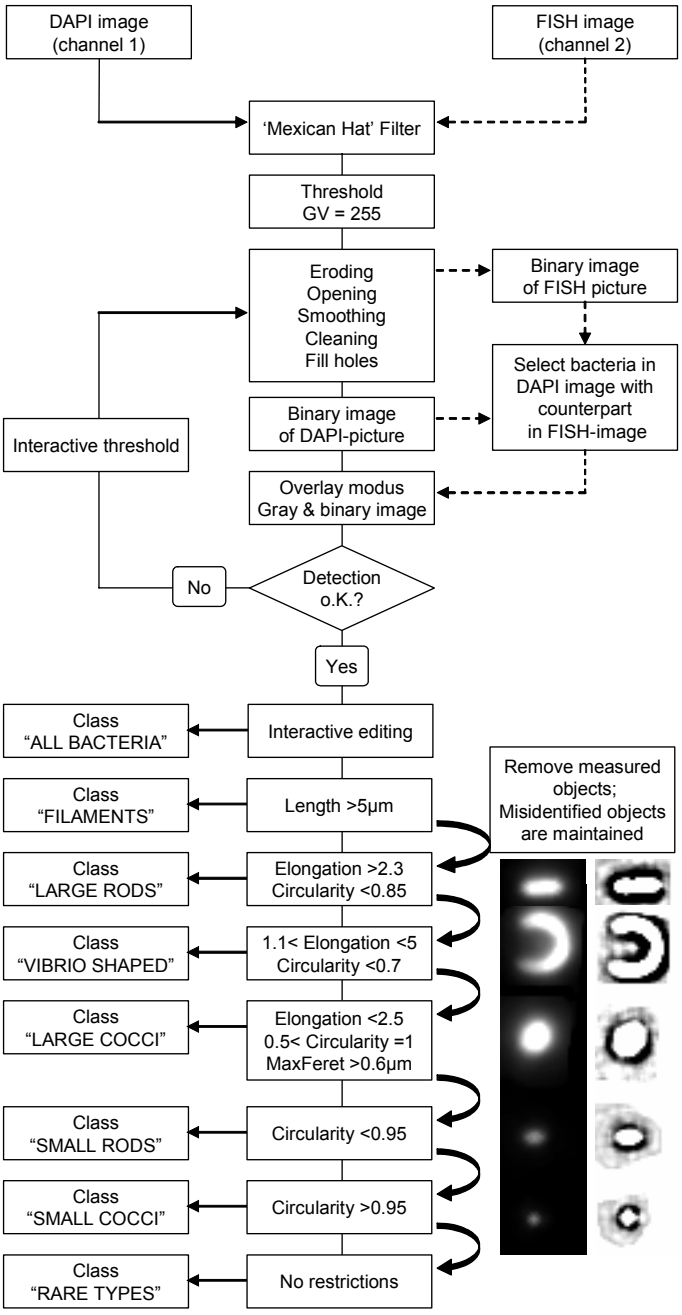

Fig. 1, Posch et al.

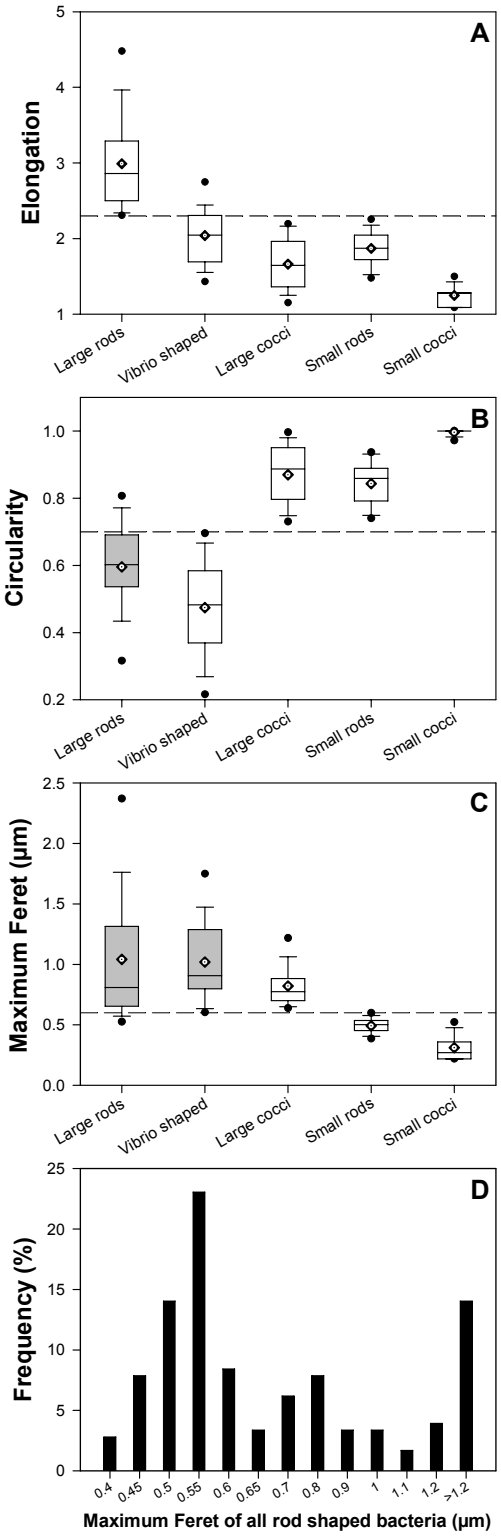

Fig.2, Posch et al.

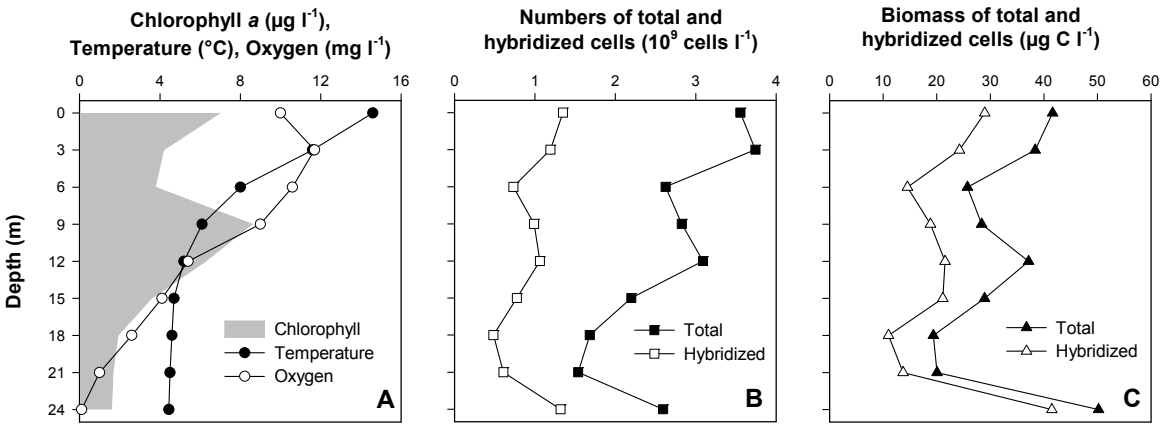

Fig. 3, Posch et al.

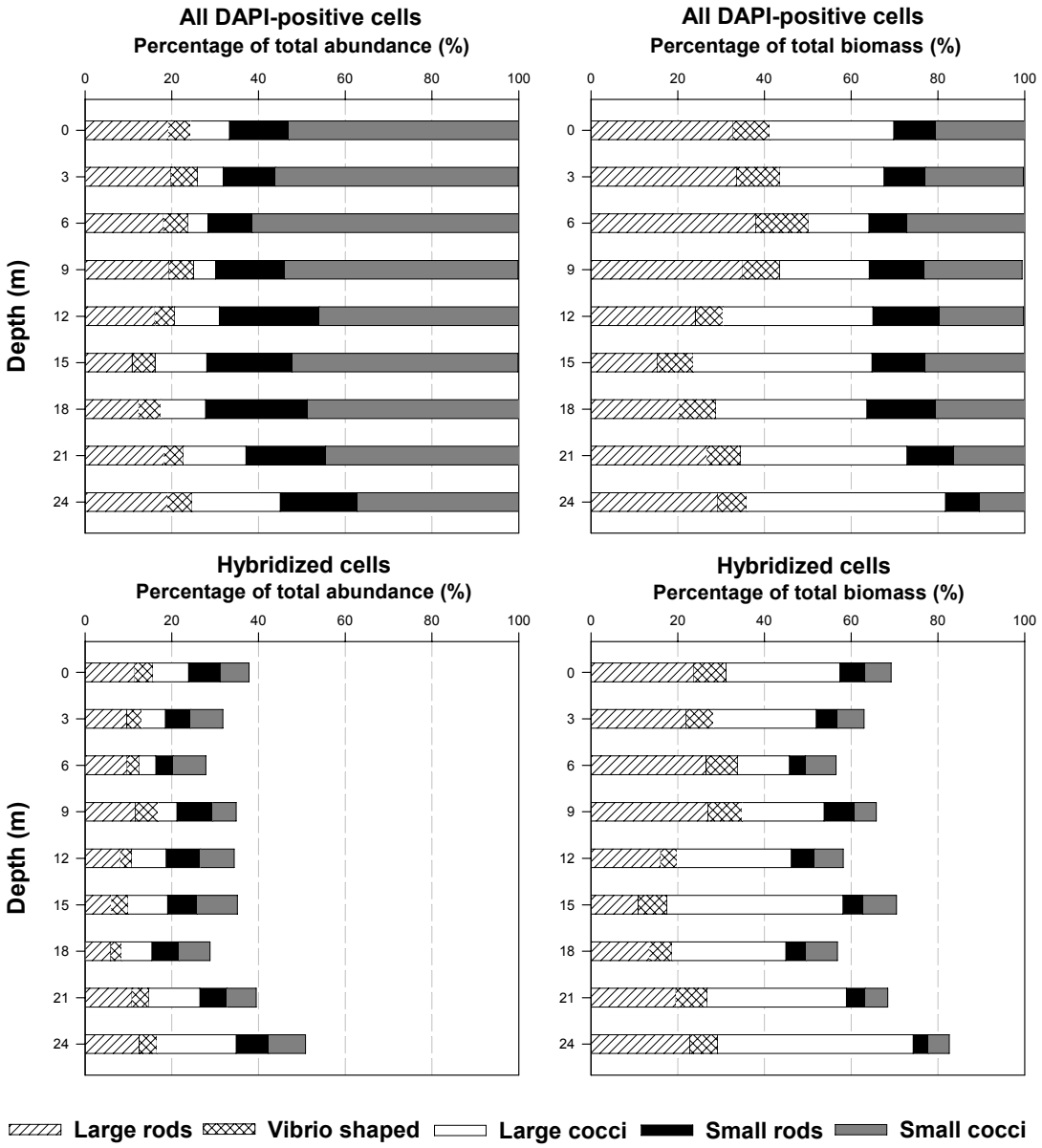

Fig. 4, Posch et al.

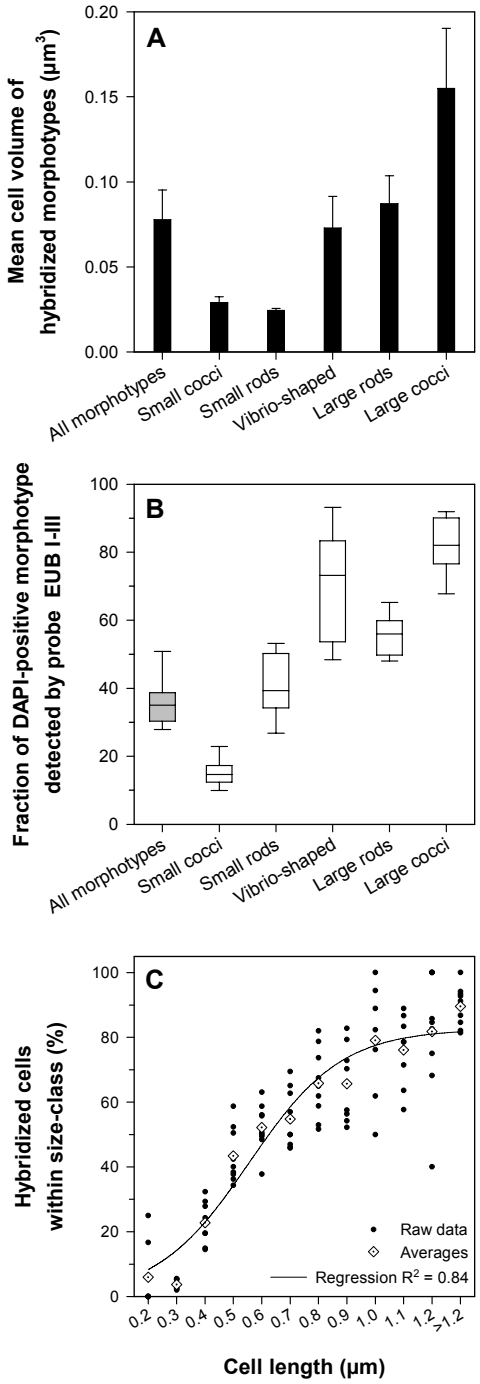

Fig. 5, Posch et al.

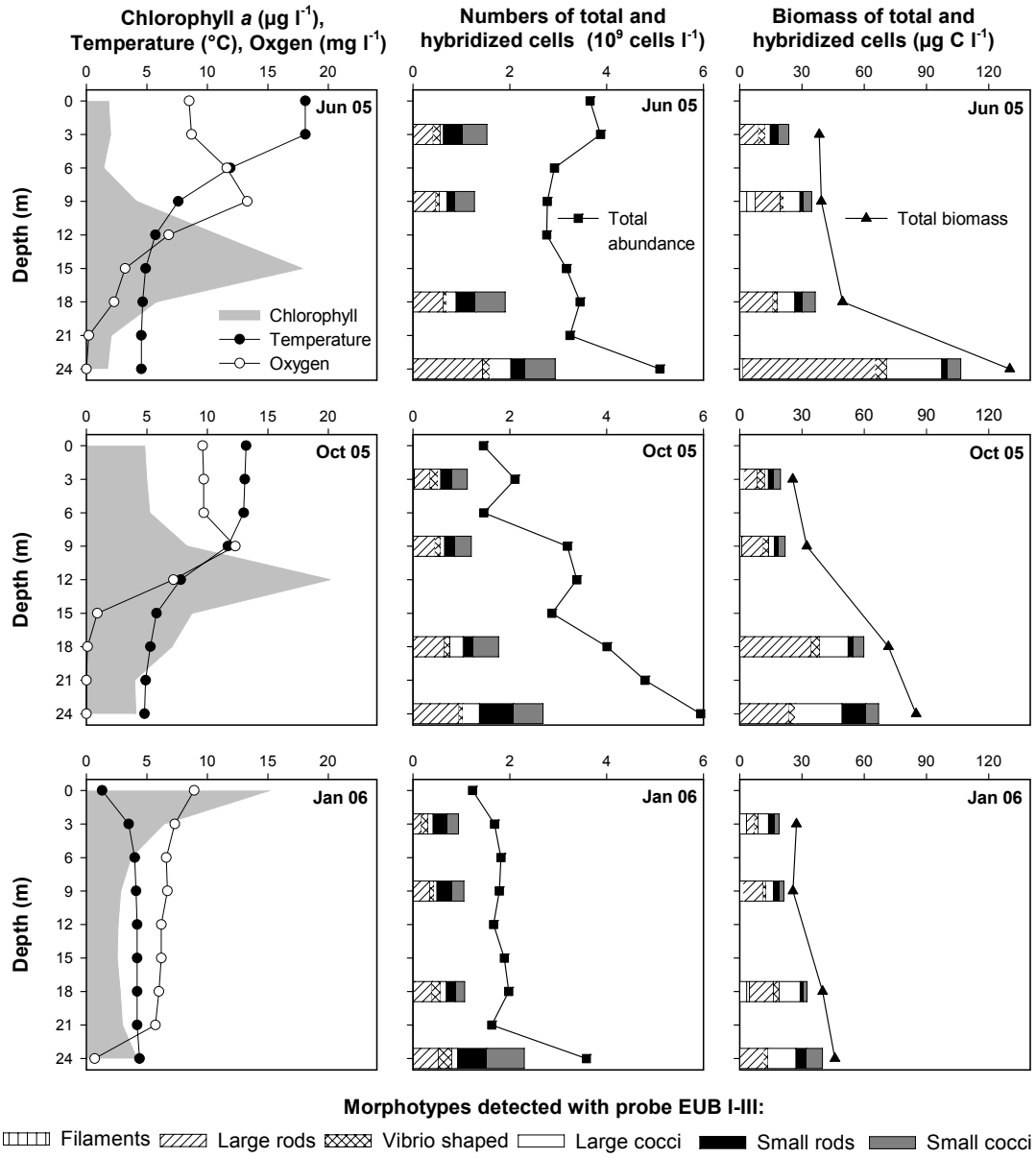

Fig. 6, Posch et al.

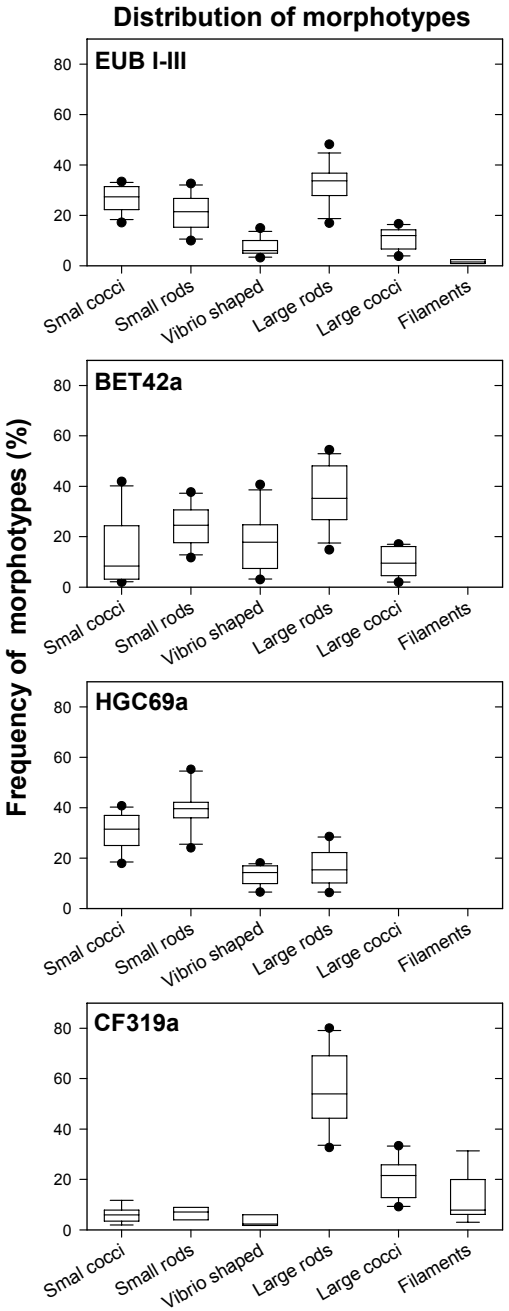

Fig. 7, Posch et al.

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Niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake

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Niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake

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Abstract

We investigated the diversity of planktonic *Betaproteobacteria* and the seasonal population changes of betaproteobacterial taxa in an oligo-mesotrophic lake (Piburger See, Austria). Focus was put on the vertical distribution of the investigated populations and on differences between their respective cell fractions with visible amino acid incorporation. On average 66% of betaproteobacterial cells and 73% of their diversity could be attributed to three lineages that were further analyzed by fluorescence *in situ* hybridization. The numbers of bacteria from the R-BT subclade of the beta I lineage and from the PnecB subgroup of the beta II cluster were rather constant throughout the water column. In contrast, members of another subgroup of beta II (PnecC) and bacteria related to *Methylophilus* (beta IV) were particularly numerous in the oxygen-depleted zone. In general, only moderate seasonal changes in abundance were observed in the upper water layers, whereas there was a clear relationship between decreasing oxygen levels and the rise of bacteria from the PnecC and beta VI clades in deeper strata. On average, almost 80% of beta I bacteria but <15% of cells from the beta IV clade showed visible amino acid incorporation. Our results suggest that the studied populations occupy distinct vertical and ecophysiological niches in Piburger See.

Introduction

Seasonality of freshwater bacterioplankton at the community level has been the topic of numerous studies. It has been shown that bacterial abundances, biomasses, and bulk production may follow seasonal patterns of phytoplankton development and/or physicochemical changes (e.g. Coveney & Wetzel, 1995; Simon et al., 1998). However, the seasonal successions of lake bacterioplankton are much less understood than of phyto- or zooplankton (Sommer et al., 1986). This is mainly due to the limited information on temporal changes of the taxonomic composition of freshwater microbial assemblages.

There is, however, first evidence that different freshwater bacteria exhibit specific seasonal succession patterns. For example, a species-like clade within *Betaproteobacteria* showed pronounced summer maxima of population sizes that were tightly correlated with water temperature (Hahn et al., 2005; Wu & Hahn, 2006a; 2006b). *Actinobacteria* from a typical freshwater lineage may form spring and autumn maxima in temperate lakes (Burkert et al., 2003; Allgaier & Grossart 2006), and a rise of filamentous bacteria affiliated with *Saprospiraceae* was observed in parallel with the decay of phytoplankton spring bloom (Schauer et al., 2006). Short-lived peaks of filamentous morphotypes related to *Sphingobacteria* and *Alphaproteobacteria* were also found during periods of high densities of bacterivorous nanoflagellates (Pernthaler et al., 2004; Nishimura & Nagata, 2007).

One drawback of many seasonal studies is the exclusive focus on the surface water layers (e.g. Burkert et al., 2003; Wu & Hahn, 2006a). Thermally stratified lakes typically exhibit pronounced depth gradients of physicochemical parameters, and higher abundances and biomasses of microorganisms can be found in deeper water layers (Cole et al., 1993). The oxygen-depleted hypolimnion may represent a niche for specialized microorganisms such as

fermenting bacteria, denitrifiers, methanotrophs, methylotrophs, or autotrophic sulfure bacteria (Casamayor et al., 2000; Lehours et al., 2007). Moreover, some heterotrophic bacteria from the oxic layers may be capable of a facultative anaerobic metabolism (Alonso & Pernthaler, 2005). Such ecophysiological questions can be addressed by combining phylogenetic identification with the assessment of single cell activity (Lee et al., 1999).

Betaproteobacteria are a prominent group of freshwater bacterioplankton that may form high abundances in lakes of different trophic status (Glöckner et al., 1999; Wu et al., 2006). Several authors have defined four to six phylogenetic clusters of freshwater *Betaproteobacteria* (Glöckner et al., 2000; Zwart et al., 2002). So far, research has mainly been focused on the ecology of bacteria from the beta I (also called *Rhodoferrax* and GKS16) and beta II (also called *Polynucleobacter necessarius*) clades (Šimek et al., 2001; 2006; Burkert et al., 2003; Wu & Hahn, 2006a; 2006b; Wu et al., 2006; Pérez & Sommaruga, 2006; 2007). In contrast, less is known about the cosmopolitan betaproteobacterial clusters beta III and beta IV (Glöckner et al., 2000).

We investigated the diversity of *Betaproteobacteria* and the seasonal abundance fluctuations of different betaproteobacterial lineages in a prealpine softwater lake in the context of changes in physicochemical parameters and chlorophyll *a* concentrations. Since this lake is characterized by stable summer stratification and an anaerobic hypolimnetic layer, focus was also put on vertical distribution patterns. In addition, potential differences between the studied populations in their preferences for amino acids were assessed.

Results

Seasonal changes of water chemistry and chlorophyll a

Piburger See was ice-covered from the beginning of our study in February 2005 until March 2005 and again from December 2005 until the end of the investigation period in February 2006. Spring mixis occurred in April 2005, but the oxygen profiles suggested only partial hypolimnetic oxygen replenishment. The lake was stratified between May and November 2005, and the metalimnion extended from 3 m depth in May to 12 m depth in November (Fig. 1). Oxygen was strongly depleted in the hypolimnion during stratification, and the anoxic water body extended up to a depth of 18 m in November 2005 (Fig. 1). Autumnal mixis occurred in early December and the aerobic zone expanded to at least 21 m depth in January 2006. Dissolved phosphorus concentrations ranged from 1.7 µg l^{-1} in 3 m in August to a maximum of 40.9 µg l^{-1} in 24 m depth in March. Nitrate was presumably consumed by algae in the upper layers of the lake during the growing season, whereas its concentrations in the hypolimnion strongly decreased with oxygen depletion (Fig. 1), concomitant with an accumulation of ammonium during late summer and autumn (data not shown).

Two pronounced peaks of chlorophyll *a* concentrations were observed in June-July and September-November, respectively. The first maximum was mainly formed by *Chrysophyceae*. A second, late autumn phytoplankton bloom almost exclusively consisted of the diatom *Asterionella* sp..

Diversity and phylogenetic affiliation of freshwater Betaproteobacteria

Altogether 39 nearly full-length and 8 partial 16S rRNA gene sequences affiliated with *Betaproteobacteria* were obtained in four clone libraries from different sampling depths and dates. Three almost complete sequences were subsequently identified as chimeras and excluded from further analysis. Our sequencing effort was sufficient to sample a high proportion of the total diversity of *Betaproteobacteria* in the libraries at 98% of sequence similarity. This was indicated by (i) a high value of Good's coverage (>0.9) and (ii) a stable estimate of total predicted phylotypes (Chao1 estimator: 13 predicted vs. 11 observed) after the analysis of at least 30 sequence types.

The majority of sequences (55%) were related to two well-separated branches within a phylogenetic lineage that is targeted by FISH probe MET1217 (Fig. 2). One of these subgroups (beta IVa) exclusively consisted of sequence types from the pelagic zone of freshwaters, whereas the other one (beta IVb) contained sequence types from a variety of habitats (wastewater treatment pools, groundwater, sediments, soil, ect.), but none from the water column. Nine percent of our sequences fell into the branch of beta I bacteria detectable by probe R-BT065 and 9% into the beta II clade (targeted by probe Beta2-870). All clones affiliated with the beta II clade were related to the species-like subcluster PnecC and targeted by probe PnecC-445. Of the remaining 12 sequences, two were affiliated with the beta III clade (GKS98 cluster), and the rest with the beta I lineage, albeit not detectable by probe R-BT065: Two sequences were related to the *Rhodoferax* sp. BAL47 clade (Zwart et al., 2002), two to *Coccomonas*, four to *Rhodoferax fermentans*, and one to the GKS16 clade (Zwart et al., 2002) and to *Leptothrix*, respectively.

Seasonal and vertical distribution patterns of different lineages of Betaproteobacteria

Between 34 to 100% (mean: 66%) of all *Betaproteobacteria* could be assigned to three phylogenetic groups by FISH with specific oligonucleotide probes. Microbes of the R-BT branch of the beta I lineage formed one third of all *Betaproteobacteria* in 3 and 9 m depth, but their contribution was significantly smaller (16%) in the hypolimnetic layers (Tables 1, 2). By contrast, the fractions of *Betaproteobacteria* affiliated with the beta II clade increased with depth, from 19% (3 m depth) to 27% (24 m depth). This was even more pronounced for beta IV bacteria, which formed only a small proportion of *Betaproteobacteria* (5%) in the epi- and metalimnion, but almost 40% in the anoxic layer.

Members of the R-BT sublineage of the beta I clade were present throughout the whole water column (Fig. 3) where they constituted between 1.4 and 7.6% of total (DAPI) cell counts. These bacteria showed seasonal fluctuations in the epilimnion with two maxima between April and June (3 m) and October to November (3 and 9 m). Distinctly higher abundances of R-BT bacteria were observed in the anoxic hypolimnetic water body (24 m) during summer stratification (June and July).

Bacteria from the beta II and beta IV clades (Probes Bet2-870 and MET1217) were both most abundant in hypolimnetic, oxygen-depleted waters (Figs. 4, 5). Members of the beta II clade occurred in rather low densities in the oxygenated water column (Fig. 4). In 18 m depth their numbers increased approximately threefold in parallel to the progressing oxygen depletion

between June and July (Fig. 1). Here, they maintained high abundances until November and subsequently decreased to approximately initial numbers (0.6×10^5 cells ml^{-1}). In 24 m, beta II bacteria increased in numbers between May and June and remained stable thereafter. Virtually all bacteria hybridized with probe Bet2-870 could be assigned to the sum of two specific probes for two subclades within beta II (PnecB, PnecC). Moreover, clear differences in the vertical distribution patterns of the two sublineages were observed. While bacteria affiliated to PnecB and PnecC occurred in roughly equal abundances in the oxygenated water column, the bloom of beta II bacteria in the sub- and anoxic layers of Piburger See could be almost entirely attributed to microbes related to PnecC (Fig. 4).

A similar trend as observed for PnecC but even more pronounced was found for microbes affiliated with the beta IV lineage (Fig. 5). In the epi- and metalimnion (3-9 m) these bacteria were close to the lower limit of precise quantification by our FISH approach (i.e., approximately 1% of total counts) throughout the investigation period (mean ± standard deviation, $0.15 \pm 0.1 \times 10^5$ cells ml^{-1}). Beta IV bacteria increased in numbers by >20 times in 18 m depth between May and June, maintained high densities until November and sharply declined thereafter. By contrast, bacteria from this lineage were always abundant in 24 m depth (between $2.6 – 5 \times 10^5$ cells ml^{-1}), forming distinct maxima in early summer and late winter (June and February, respectively).

Incorporation of amino acids by different groups of Betaproteobacteria

On average, 76% of all *Betaproteobacteria* with visible incorporation of amino acids were members of the three studied subgroups. While beta I and beta IV bacteria showed only slight changes in amino acid uptake activity, microbes of the beta II lineage exhibited pronounced temporal and vertical fluctuations (Table 2, Figs 3, 4, 5).

Bacteria from the beta I clade had significantly higher fractions of active cells than the other two groups (ANOVA, $p<0.001$), and on average 79% (min: 52%, max: 94%) of cells hybridized by probe R-BT065 incorporated amino acids (Fig. 3). These bacteria represented >60% of all active *Betaproteobacteria* in the upper layers (3 and 9 m depth), but only approximately one third in the hypolimnion (18 and 24 m depth). Bacteria from the beta II clade showed the opposite trend, with higher contributions to active *Betaproteobacteria* in 24 m depth (30%) than in the epilimnion (16%). On average, approximately one third of these bacteria incorporated amino acids (mean: 36%, min: 13%, max: 70%) (Fig. 4). The highest fractions of active beta II cells (mean 53.6%) were found between June and November in 18 m, and between June and February in 24 m depth, respectively. Due to the low abundances of beta IV bacteria in the upper water body it was not possible to determine MAR-positive fractions of these bacteria in 3 and 9 m depth. Members of this lineage showed significantly (ANOVA, $p<0.001$) lower amino acid uptake activity (mean 13%, min: 7%, max: 20%) than the other studied groups in 18 and 24 m. Consequently, their contribution to total MAR-positive *Betaproteobacteria* was low.

Relationship between environmental parameters and distribution patterns of different Betaproteobacteria

The first two axes of the RDA model explained 99.1% of the variability in abundance and amino acid uptake of the different betaproteobacterial groups (Fig. 6). Axis 1 explained 93% of the variability and was found to correlate most highly with oxygen concentrations (r=-0.85) and dissolved phosphorus (r=0.68). The second axis explained only 6.1% of the variability. Dissolved organic carbon and chlorophyll *a* concentrations were identified as the most important second axis variables (r=-0.40 each). Population sizes of beta IV and PnecC bacteria were clearly separated from R-BT and PnecB bacteria on the first axis of RDA, and from each other on the second one. By contrast, the abundances of R-BT bacteria were mainly related to the second axis, where they aligned with PnecC bacteria. RDA did not significantely explain changes in the fractions of active cells in either R-BT or beta II bacteria.

Discussion

Occurrence of Betaproteobacteria *as detected by FISH and clone libraries*

Recently, Cottrell and coworkers (2005) compared microbial diversity in PCR based and metagenomic clone libraries from a river. Sequences related to the beta II and beta IV clades were only detected in the PCR based libraries, whereas beta I sequences were found in both. The authors asked for additional FISH data with probes specific for the beta II and beta IV lineage to resolve their numerical importance. Our study illustrates that bacteria from all three clades were common in Piburger See (Figs 3, 4, 5).

While over 80% of all *Betaproteobacteria* could be hybridized by the applied set of probes in the anoxic water body of Piburger See, FISH coverage was substantially lower (60%) in the epi- and metalimnetic samples. Thus other *Betaproteobacteria* might also form large populations in the lake at least seasonally. This is also suggested by sequence analysis: Approximately one quarter of the sequence types in our 16S rRNA gene clone libraries (mainly from other branches of the highly diversified beta I lineage) were not detected by the specific FISH probes (Fig. 2). Two sequence types from 9 m depth untargeted by our FISH probes were related to the beta III (GKS98) clade. These bacteria were originally described from a softwater high mountain lake (Pernthaler et al., 1998), but are widely distributed across freshwater systems (Zwart et al., 2003; Wu et al., 2006). Interestingly, bacteria from the species-like PnecB lineage of beta II could be detected by FISH throughout the investigation (Fig. 4). However, no corresponding PnecB genotypes were present in our sequence collection, although different indices suggested that most betaproteobacterial diversity in the libraries at the species level had been covered.

Substrate incorporation patterns of different Betaproteobacteria

The three studied betaproteobacterial populations clearly differed in their respective incorporation of radiolabeled amino acids (Table 2, Fig. 3, 4, 5). In the epi- and metalimnion the fractions of active bacteria from the R-BT clade were always larger than from the beta II clade irrespective of, e.g., changes in water temperature or DOC concentration, and there were always

less MAR-positive cells in the beta IV than in the beta II lineage in anoxic waters. This suggests that there might be consistent, growth-rate independent differences in the preference of the three populations for amino acids characteristic of their respective ecophysiological niches.

Unfortunately it was not possible to incubate MAR samples at strictly ambient oxygen conditions in the suboxic and anoxic layers of Piburger See (18-24m). Therefore, the high fractions of MAR-positive cells from the beta I and II lineages (Figs 3, 4) might not accurately reflect their *in situ* levels of activity, but rather indicate their potential for a facultatively anaerobic lifestyle. Since amino acid uptake is dependent on ATP-driven transporter systems (Hosie & Poole, 2001), oxygen-independent energy sources might be used by these bacteria for the incorporation of the offered substrates. A facultatively anaerobic metabolism of beta II and beta IV bacteria in 18 m depth was also suggested by the rather constant percentages of active cells in this layer throughout the season irrespective of the drastic changes in ambient oxygen concentrations (Table 2, Figs. 1, 3, 5).

Members of the R-BT subclade of Betaproteobacteria

All sequences affiliated with the R-BT subcluster (and detected by probe R-BT065) originated from clone libraries from 3 or 9 m depth, suggesting that bacteria from this widespread group of freshwater bacteria (Zwart et al., 2002; Glöckner et al., 2000) preferably inhabited the oxygenated layers of Piburger See. However, R-BT bacteria were also present and highly active in hypolimnetic layers of the lake (Fig. 3). This hints at high ecological plasticity within this lineage, as has been observed for other freshwater *Betaproteobacteria* (Hahn, 2006).

The population sizes of R-BT bacteria in Piburger See showed some seasonal variability both in the epi- and hypolimnion (Fig. 3). This was also reflected in their alignment with the second axis of redundancy analysis (Fig. 6), which is most strongly related to seasonal parameters such as chlorophyll *a* and DOC concentrations (Fig. 1). Bacteria detectable with probe R-BT065 are preferably ingested by heterotrophic nanoflagellates (Jezbera et al., 2006), and they are typically enriched if protistan predators are eliminated via size-fractionation (Šimek et al., 2001; 2005) or dilution (Pérez & Sommaruga, 2006). In fact, these microbes may compensate predation mortality by their unusually high growth rates (Šimek et al., 2001; 2005; Horňák et al., 2006). R-BT bacteria were observed to rapidly multiply after addition of phosphorus (Šimek et al., 2006; Salcher et al., 2007), algal derived DOC (Pérez & Sommaruga, 2007), or if transplanted from nutrient-poor to nutrient-richer environments (Šimek et al., 2006). Altogether these findings indicate that microbes from this phylogenetic lineage follow an "opportunistic" life strategy i.e., that they respond to environmental changes by rapid increase in growth rates, but are also tightly controlled by predation.

Beta II subgroup of Betaproteobacteria

Bacteria related to the beta II lineage have been found in acidic, alkaline and neutral freshwater habitats in different climatic zones (Hahn, 2003; Wu et al., 2006). Water chemistry, in particular pH and salinity, has been identified as an important factor for the ecological

differentiation of two subclusters within this lineage, PnecB and PnecC (Wu & Hahn, 2006a, b). Beta II bacteria appear to be readily culturable, e.g. by gradual acclimatization to rich media (Hahn, 2003). Numerous strains of free-living, aerobic, heterotrophic ultramicrobacteria from this lineage (predominantly from the PnecC and PnecD subclades) have been isolated from various freshwaters (Hahn, 2003; Hahn et al., 2005; Wu & Hahn, 2006a; Vannini et al., 2007). The generally small cell sizes of beta II bacteria have also been confirmed *in situ* (Wu & Hahn, 2006a), and some strains seem to range at the lower size-dependent uptake limit of heterotrophic nanoflagellates (Boenigk et al., 2004).

In the epi- and metalimnion of Piburger See, the subgroups PnecB and PnecC each approximately represented half of all beta II bacteria, respectively (Fig. 4). Microbes related to PnecB were present in low numbers (0.2-1.5% of total cells, Table 2) throughout the year in all depth layers, and no pronounced seasonality could be observed. In contrast, distinct seasonality of PnecB bacteria was found in surface waters of Lakes Mondsee and Taihu, with maxima in summer and winter, respectively (Wu & Hahn, 2006a). The species-like PnecC subcluster of the beta II clade harbors ecologically distinct free-living strains as well as phylogenetically closely related obligate endosymbionts of the freshwater ciliate *Euplotes* sp. (*Polynucleobacter necessarius*) (Hahn, 2003; Vannini et al., 2007). PnecC bacteria may persistently occur in humic lakes (Newton et al., 2006), where they can form pronounced seasonal fluctuations (Burkert et al., 2003) and transient blooms of up to 60% of total cells (Hahn et al., 2005). However, members of PnecC are widespread across various freshwaters and have, e.g., also been reported to form seasonal blooms in a shallow eutrophic subtropical lake (Wu & Hahn, 2006a).

So far, there is no information about the vertical distribution of PnecC bacteria in freshwaters. Our findings thus expand the current knowledge about the ecology of PnecC bacteria by illustrating their potential preference for oxygen-depleted and anoxic waters: Members of the PnecC lineage formed a distinct summer maximum in the hypolimnetic waters (18 m) of Piburger See, and they were significantly more numerous at 24 m depth than in the two epi- and metalimnetic layers (Fig. 4). Moreover, >50% of all cells affiliated to beta II from anoxic waters (and thus presumably to PnecC) readily incorporated amino acids, indicating *de novo* biomass production by these bacteria. It is, therefore, likely that members of the PnecC clade can either use other electron acceptors than oxygen or that they are capable of fermentation.

Bacteria affiliated with the beta IV lineage

All 16S rRNA gene sequences from the beta IV lineage obtained from Piburger See fell into two well-separated branches (Fig. 2). One clade (beta IVa) exclusively contains freshwater sequences of the beta IV (Glöckner et al., 2000) or LD28 cluster (Zwart et al., 2002), as well as all of our sequences from 3 and 9 m depth (plus one from 24 m). All but one of the other betaproteobacterial sequences in our 24 m clone library were affiliated with a second lineage (beta IVb) that harbors sequences from habitats such as wastewater treatment plants, groundwater, subsurface water, mine drainage water, sedimentary rocks, lake sediment, or oil polluted soils (e.g. Connon et al., 2005; Battaglia-Brunet et al., 2006). The closest taxonomically

described relative of beta IV bacteria is *Methylophilus methylotrophus*. It is an obligate type I methylotrophic bacterium, isolated from activated sludge (Jenkins et al., 1987) which can grow only on one-carbon organic compounds such as methanol or formaldehyde, but does not use methane as substrate (Anthony, 1982). Other cultured bacteria closely related to the freshwater beta IV lineage are also known to be methylotrophs (e.g. *Methylobacillus* sp. (Yordy & Weaver, 1977), *Methylovorus* sp. (Govorukhina & Trotsenke, 1991)). Members of the family *Methylophilaceae* are important denitrifyers in wastewater treatment plants and some of these bacteria use methanol as a substrate *in situ* (Ginige et al., 2004). Stable isotope probing in lake sediment microcosms with the ^{13}C labelled C_1 substrates methanol, methylamine and formaldehyde selectively yielded sequence types related to *Methylophilaceae* (Nercessian et al., 2005).

The FISH probe MET1217 was independently designed by Friedrich et al. (2003) and Ginige et al. (2004) for studying bacterial communities in an industrial biofilter and in an anoxic methanol-fed sequencing batch reactor, respectively. CARD-FISH revealed a distinct vertical pattern of these bacteria in Piburger See (Fig. 5). While cells hybridized by probe MET1217 were almost absent in the epi- and metalimnion, these bacteria formed the dominant fraction of *Betaproteobacteria* in the hypolimnion (Table 2, Fig. 5). Due to the rather broad specificity of the probe (Fig. 2) it remains unclear if bacteria from both branches of beta IV shared the same vertical distribution. Recently, eight strains of the beta IVa cluster have been isolated from three German lakes on a mix of organic substrates under aerobic conditions (Gich et al., 2005), indicating that these bacteria might be more important in epilimnetic waters. By contrast, all but two 16S rRNA gene sequences from our 24 m library were from the beta IVb lineage, and other sequences from this subclade were mainly obtained from anoxic habitats (Fig. 2). Therefore, we hypothesize that predominantly methylotrophic bacteria affiliated with the beta IVb lineage were abundant in the suboxic and anoxic layers of Piburger See. This is also supported by the low fractions of cells hybridized with probe MET1217 that could incorporate amino acids (Fig. 5).

Conclusions:

The activity of R-BT bacteria and the numerical importance of beta IV bacteria in anoxic waters as well as the vertical niche separation of PnecB and PnecC in Piburger See would have been missed by sampling of the upper layers of the lake only. Thus, a vertical profiling of the anoxic zones of freshwaters might substantially expand our knowledge of which bacteria are typical components of lake bacterioplankton and it may also change our view on the physiology of common lake bacteria.

Experimental procedures

Study site and sampling

Piburger See is an oligo-mesotrophic dimictic lake situated in the Tyrolean Alps, Austria, at 913 m a.s.l. Further information on the lake can be found elsewhere (Tolotti & Thies, 2002). Water samples were taken monthly over a period of one year (from February 2005 to February 2006) at

the deepest part of the lake (24.6 m) from 3, 9, 18 and 24 m depths with a 5 l Schindler-Patalas sampler. Water temperature was directly determined during sampling. No samples could be collected in December 2005 due to the unstable ice coverage of the lake.

Portions of 40 ml from each sample were fixed either with formaldehyde (2% final concentration) for bacterial abundance determination, or with freshly prepared buffered paraformaldehyde (pH 7.4, 2% final concentration) for analysis by CARD-FISH. All fixed samples were stored at 4°C until further processing. In addition, unfixed water samples were collected for MAR-FISH analyses (40 ml), for the construction of 16S rRNA gene clone libraries (1 l), and for chemical analyses (2 l). These samples were delivered to the lab at *in situ* temperature within 2 hours. The following chemical parameters were evaluated: Oxygen concentrations (mg l^{-1}), concentrations of chlorophyll *a* (Chl*a*, µg l^{-1}), nitrate (NO_3^-, µg l^{-1}), dissolved phosphorus (DP, µg l^{-1}), and dissolved organic carbon (DOC, mg l^{-1}). Samples for phytoplankton analysis (100 ml) were fixed with Lugol's solution and counted with an inverted microscope with phase contrast.

Bacterial abundances

Two to 2.5 ml of formaldehyde-fixed samples were stained with DAPI (4',6-diamidino-2-phenylindole, 6.7 µg ml^{-1} final concentration, Porter & Feig, 1980), filtered onto black polycarbonate filters (Osmonics, 0.22 µm pore size, 25 mm diameter), and evaluated with an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss, Germany). At least 1000 bacteria (Zeiss filter set 01) were counted per sample at a total magnification of 1600 ×.

16S rRNA gene clone libraries and phylogenetic sequence analysis

Unfixed water samples were prefiltered through 3 µm to remove larger organisms. Subsequently, 300 – 600 ml were filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2 µm pore size, 47 mm diameter). Filters were air dried and stored at -80°C until further processing. Four preparations from different periods (April and May 2005) and depths (3, 9 and 24 m) were processed to produce 4 clone libraries. Small pieces of the filters were used as template for PCR as previously described (Kirchman et al., 2001) using the primers GM3F and GM4R (Muyzer et al., 1995). PCR products were purified with the QIAquick PCR purification kit (QIAGEN), inserted into TOPO vectors (TOPO TA cloning kit for sequencing; Invitrogen) and cloned into competent cells of *E. coli* according to the manufacturer's instructions. After screening of the clones for right-sized inserts, plasmid preparations were done with the QIAprep Spin Miniprep Kit (QUIAGEN) or with the Montage™ Plasmid Miniprep96 Kit (Millipore). The sequencing reactions were accomplished with the M13F vector primer and the ABI BigDye chemistry on an ABI 3130x Genetic Analyzer (Applied Biosystems). Partial sequences were first analyzed with the BLAST queuing system (www.ncbi.nlm.nih.gov/BLAST/) for their phylogenetic affiliations. Nearly full-length sequences of all unique sequence types affiliated with *Betaproteobacteria* were obtained by additional sequencing with vector primer M13R (Messing, 1983) and primer GM1 F (Muyzer et al., 1993).

Partial sequences were assembled with the Sequencher software (Gene Codes) and checked for chimeric origin using the software Pintail (Ashelford et al., 2005). Phylogenetic analyses were performed with the ARB software package (Ludwig et al., 2004). The ARB database was complemented with betaproteobacterial sequences deposited in GenBank that were closely related to our sequences. All sequences were first automatically aligned using the ARB tool FAST_ALIGNER and alignments were subsequently manually optimized. For the reconstruction of phylogenetic trees, only nearly full-length (i.e. longer than 1400 nucleotides) sequences were considered. A 50% base frequency filter and a filter designed with betaproteobacterial sequences were used to exclude highly variable positions. Maximum-parsimony, neighbor-joining and maximum likelihood analyses were performed with the respective ARB tools. The resulting trees were compared manually to obtain a consensus tree. Partial sequences were added to this consensus tree in accordance with maximum-parsimony criteria, without allowing changes in the tree topology.

Abundances of different Betaproteobacteria

Ten to 15 ml of paraformaldehyde-fixed samples were filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2 µm pore size, 47 mm diameter), rinsed with distilled water, air dried, and stored at -20°C until further processing. CARD-FISH was carried out as previously described (Sekar et al., 2003) with horseradish peroxidase labeled oligonucleotide probes specific for *Betaproteobacteria* (BET42a, Amann et al., 1995) and three subclades within this group: R-BT065 (closely related to *Rhodoferax* sp. BAL47, belonging to the beta I cluster, Šimek et al., 2001), Bet2-870 (*Polynucleobacter necessarius*- or beta II cluster, Burkert et al., 2003), and MET1217 (order *Methylophilales*, Friedrich et al., 2003) which includes freshwater bacteria from the beta IV cluster (Glöckner et al., 2000). In addition, the probes PnecB-23S-116 and PnecC-445 (Wu & Hahn, 2006a) were used for the discrimination of two subclusters within the beta II clade. Signal amplification was performed using tyramides labeled with Alexa488 (Invitrogen). Filter sections were counter-stained with DAPI (1 µg mL^{-1}) and inspected with an epifluorescence microscope (Axiophot, Carl Zeiss) at a magnification of 1250 × and the filter sets 01 (DAPI) and 10 (Alexa 488). For manual evaluation of the fractions of hybridized cells, at least 500 to 1000 DAPI-stained cells were counted.

The majority of FISH and MAR-FISH preparations were evaluated by automated cell counting using a set of manual counts for system calibration (Pernthaler et al., 2003). Evaluations were performed with a fully automated system consisting of an epifluorescence microscope (AxioImager.Z1, Carl Zeiss) with a motorized stage for eight microscopic slides (Zeiss WSB Piezodrive 05) and a CCD Camera (AxioCam MRm, Carl Zeiss), linked to a personal computer with the image analysis software AxioVision 4.6 (Carl Zeiss). A 63 × "PlanApo" objective was used for image acquisition. Automation was achieved using the Visual Basic for Application module of AxioVision (M. Zeder, unpublished). Briefly, the microscope was loaded with up to eight slides, overview images were automatically acquired using a 1 × EC "Plan-Neofluar" objective and filter pieces were detected. A x,y-position list was generated containing a user-defined number of

locations equally distributed on each filter piece. Two images (DAPI and Alexa 488 fluorescence) were acquired at each microscopic field. After image acquisition and an automated quality control routine to discard images of low quality, a counting routine automatically processed all images and detected the fractions of hybridized bacteria within all DAPI-stained cells.

In order to better assess counting precision five duplicate hybridizations were evaluated for each probe. Only insignificant differences between duplicate determinations were observed (paired t-test, $p>0.2$).

Microautoradiography combined with CARD-FISH (MAR-FISH)

Five mL of samples were incubated with [^{3}H]-amino acids (5 nM final concentration, 48 Ci mmol^{-1} specific activity, Amersham) for 1 hour at *in situ* temperatures. After fixation with buffered paraformaldehyde (pH 7.4, 2% final concentration) duplicate subsamples of 2 ml were filtered onto white polycarbonate filters (Millipore, type GTTP, 0.2 µm pore size, 25 mm diameter), rinsed twice with sterile water and stored at -20°C until further processing. CARD-FISH of filter sections was carried out as described above. MAR-FISH was performed as previously described (Alonso & Pernthaler, 2005). Briefly, filter sections were glued onto glass slides with 2% agarose (Seakem), dipped into autoradiography emulsion (NTB emulsion, Kodak) in the darkroom, placed on ice for 10 min, and exposed in the dark at 4°C for 2-4 days. Thereafter, slides were developed with Dektol developer (Kodak) and Kodak fixer (Kodak) following the manufacturer's instructions. After drying, filters were embedded in a mounting medium containing DAPI (1 µg ml^{-1}). The evaluation of MAR-FISH filters was carried out by fully-automated image analysis as described above, but an additional stack of 9 bright field images in 0.5 µm steps above the z-position of the DAPI image was acquired to detect the silver grains (M. Zeder, unpublished). No samples for MAR-FISH were collected in January 2006, and no MAR-FISH samples were evaluated in which the fractions of probe positive cells were below 1.5% of total (DAPI) counts.

Statistical analyses

Prior to statistical analyses, all data of relative abundances (percentages) were arcsine transformed to obtain normal distribution, whereas cell numbers and chemical variables were log(x+1)-transformed. We used ANOVA to test for significant differences between the different depth layers and for differences in the amino acid uptake between betaproteobacterial clades. Furthermore, paired t-tests were carried out. The program R (www.r-project.org) was used for these statistical analyses.

The coverage of the diversity of *Betaproteobacteria* in our clone libraries (Good's C index, Good, 1953) and the estimated total diversity (S_{Chao1} estimator, Chao, 1984) were calculated using 98% of sequence similarity as cutoff for the classification of different sequence types. Analyses were performed with the online input form created by Kemp & Aller (2004).

Redundancy analysis (RDA) was used to determine the effects of environmental variables on betaproteobacterial abundances and the fraction of cells with visible amino acid incorporation (Stewart & Love, 1968). Only those environmental variables exhibiting a significant correlation

(p<0.001) to betaproteobacterial abundances or amino acid uptake were included in the analysis. Activity patterns of beta IV bacteria were excluded from analysis because of the high number of missing data points. The significance of added variables was tested by a Monte Carlo permutation test (499 permutations, p<0.001). Analyses were performed with the Microsoft EXCEL add-in program XLSTAT-ADA (www.xlstat.com).

Nucleotide sequence accession numbers

All 16S rRNA gene sequences have been deposited to EMBL under the accession numbers AM849424 to AM849467.

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Table 1: Vertical differences in the relative contributions of the studied populations to all *Betaproteobacteria* (i.e., cells hybridized with probe Bet42a) and to *Betaproteobacteria* with visible amino acid incorporation (MAR+ cells). Data were arc sine transformed prior to analysis (one-way ANOVA). F-values are given for significant differences (* p<0.01, ** p<0.005, *** p<0.001). ND: no MAR data were determined for bacteria hybridized with probe MET1217 in one of the layers; ns: not significant.

	R-BT065	Bet2-870	MET1217
Total *Betaproteobacteria*			
3 m vs 9 m	ns	ns	ns
3 m vs 18 m	**14.66*** **	ns	**18.93*** **
3 m vs 24 m	**23.28*** **	5.07*	**112.09*** **
9 m vs 18 m	**18.19*** **	ns	**15.36*** **
9 m vs 24 m	**28.53*** **	ns	**116.99*** **
18 m vs 24 m	ns	ns	13.28**
MAR+ *Betaproteobacteria*			
3 m vs 9 m	ns	ns	ns
3 m vs 18 m	**5.58***	ns	ND
3 m vs 24 m	**13.47****	4.61*	ND
9 m vs 18 m	**5.16***	ns	ND
9 m vs 24 m	**9.67****	8.24**	ND
18 m vs 24 m	ns	ns	ns

Table 2: Means and annual ranges (in brackets) of relative abundances (in % of DAPI) and of cells with visible amino acid incorporation (MAR+ cells) (in % of MAR+ DAPI) of the studied betaproteobacterial clades. ND: no MAR data were produced.

Depth	R-BT065	Bet2-870	MET1217	PnecB	PnecC
Relative abundance (% of DAPI)					
3 m	3.5 (2.3-7.6)	2.1 (0.6-3.6)	0.6 (0-1.2)	0.8 (0.3-1.5)	0.8 (0.4-1.4)
9 m	3.0 (1.4-6.2)	2.0 (1.4-2.9)	0.6 (0.1-1.3)	0.6 (0.2-1.3)	0.6 (0.2-1.2)
18 m	2.4 (1.4-3.5)	3.4 (1.2-6.6)	2.7 (0.4-6)	0.6 (0.3-0.9	2.9 (0.8-7.3)
24 m	3.4 (1.5-5.2)	5.7 (2.7-9.1)	8.7 (4.4-12.2)	0.5 (0.2-0.9)	5.6 (3.4-7.1)
Relative abundance of MAR+ (% of MAR+ DAPI)					
3 m	19.6 (7.9-	5.2 (2.9-12.9)	ND	ND	ND
9 m	14.3 (6.3-	3.4 (1.1-6.1)	ND	ND	ND
18 m	9.6 (7.7-12.6)	8.9 (1.2-22.2)	2.9 (0.7-5.2)	ND	ND
24 m	18.7 (8.3-	17.7 (5.5-28.2)	7.4 (3.7-10.7)	ND	ND

Figure legends:

Figure 1: Seasonal and vertical fluctuations of different environmental parameters in Piburger See during the study period (February 2005-February 2006).

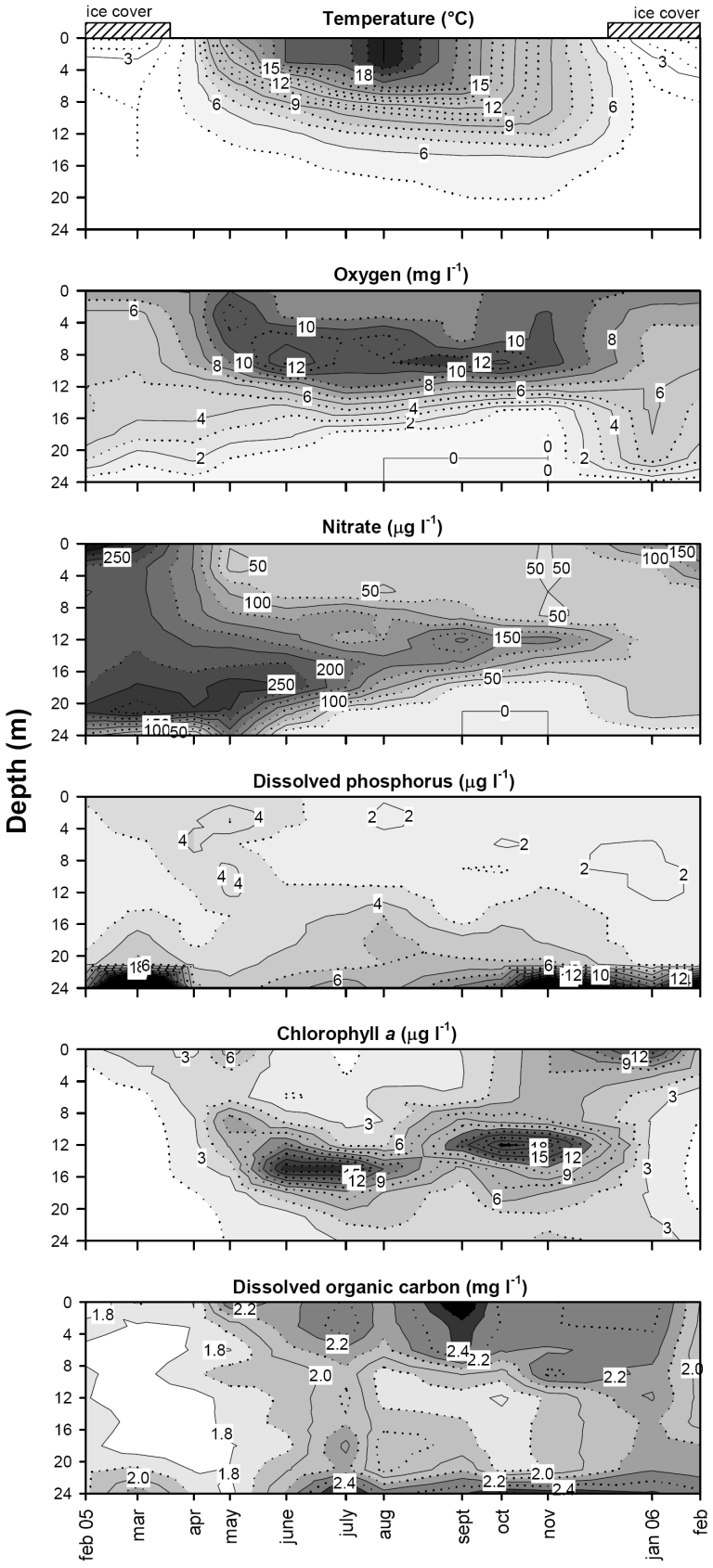
Figure 2: Phylogenetic affiliation of betaproteobacterial sequence types from four 16S rRNA gene clone libraries obtained at different time points and depths (in bold). Partial sequences are shown with dotted lines, and identical sequences (similarity 99.9% or higher) are presented in one line. The scale bar represents 10% estimated sequence divergence.

Fig. 3: Seasonal and vertical patterns of abundance (10^5 cells ml^{-1}) and amino acid uptake activity (AA uptake, in percent of all probe positive cells) bacteria from the beta I clade (as detected by probe R-BT065).

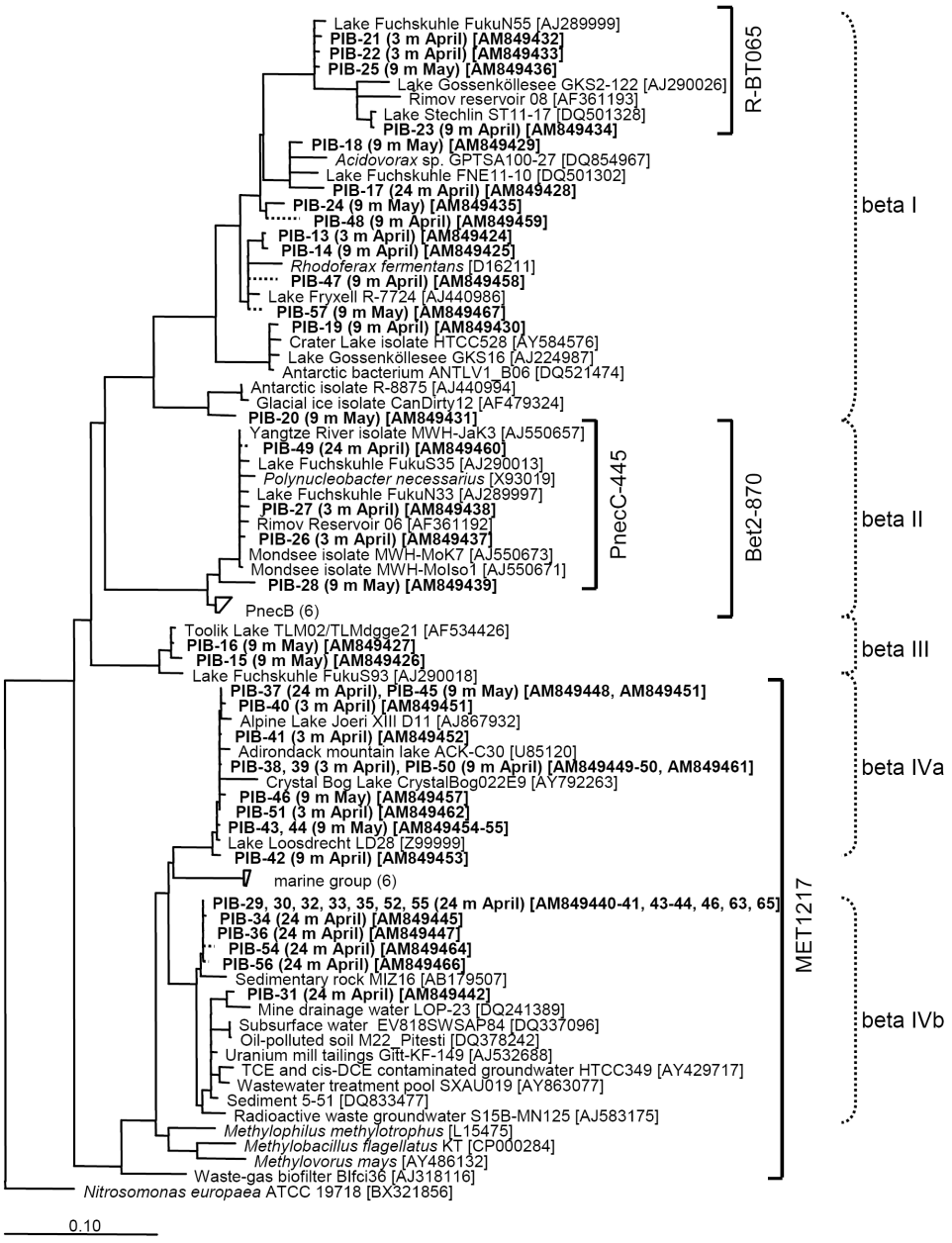
Fig. 4: Seasonal and vertical patterns of abundance (10^5 cells ml^{-1}) and amino acid uptake activity (AA uptake, in percent of all probe positive cells) bacteria from the beta II clade (as detected by probe Bet2-870, left panels) and abundances of bacteria from the PnecB and PnecC subclades of beta II (10^5 cells ml^{-1}, right panels).

Fig. 5: Seasonal and vertical patterns of abundance (10^5 cells ml^{-1}) and amino acid uptake activity (AA uptake, in percent of all probe positive cells) bacteria from the beta IV clade (as detected by probe MET1217). No MAR data were produced for fractions of probe positive cells below 1.5% of total (DAPI) counts.

Fig. 6: Redundancy analysis (RDA) biplot showing the different betaproteobacterial clades in cell numbers (□) and visible amino acid incorporation (▲) in relation to the strongest environmental variables. The eigenvalues of the two axes are given in brackets. Abbreviations: oxygen: oxygen concentrations; DP: dissolved phosphorus concentration; temp: temperature; DOC: dissolved organic carbon; Chl*a*: chlorophyll *a* concentrations; NO_3: nitrate concentration.



Salcher et al., Figure 1



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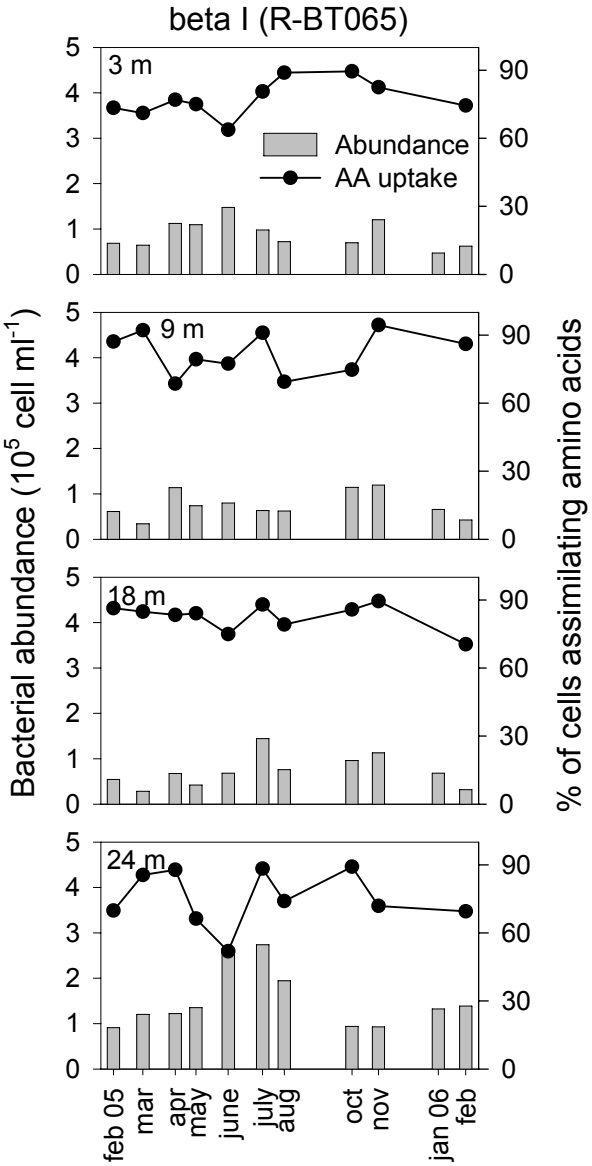
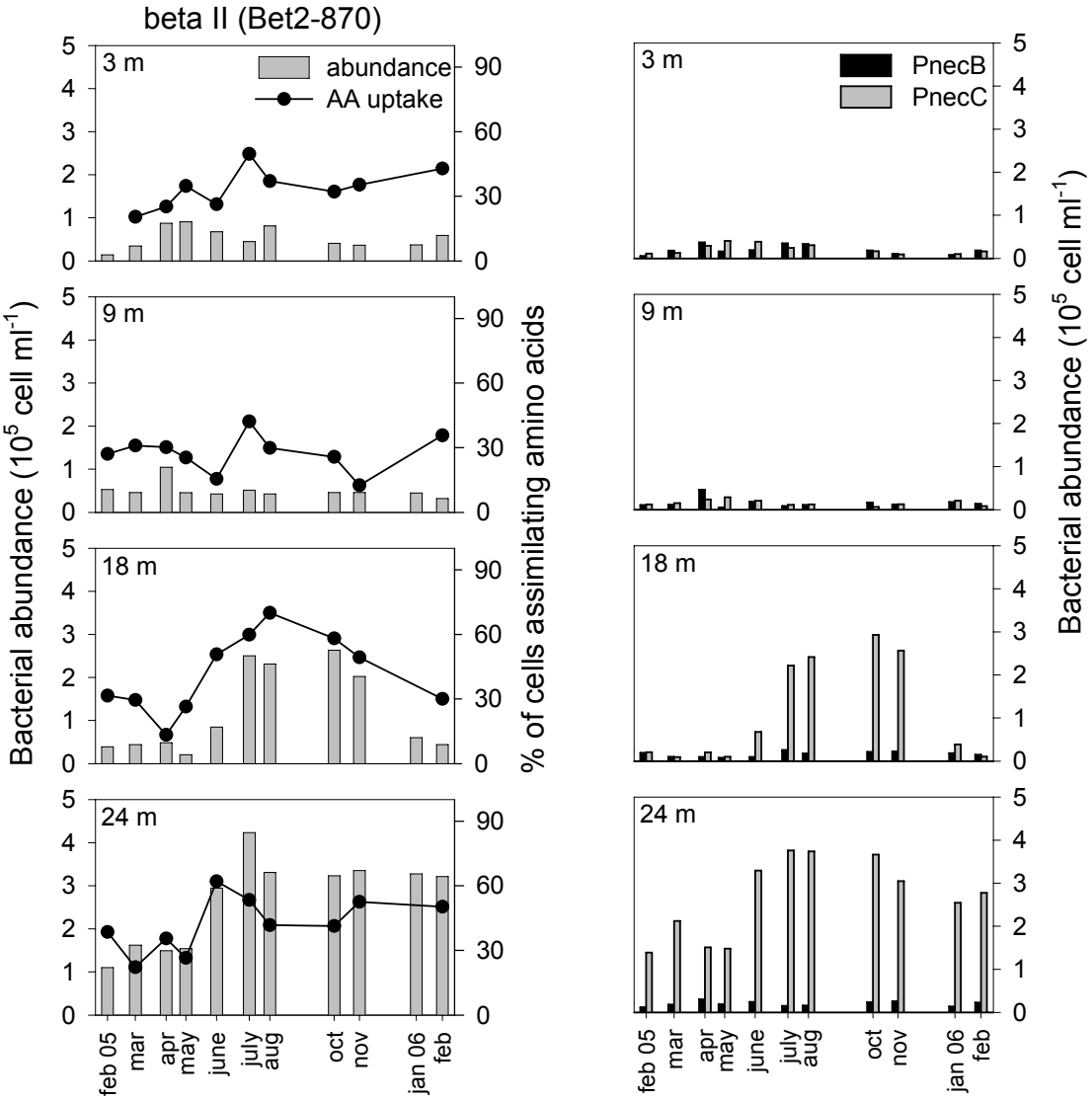
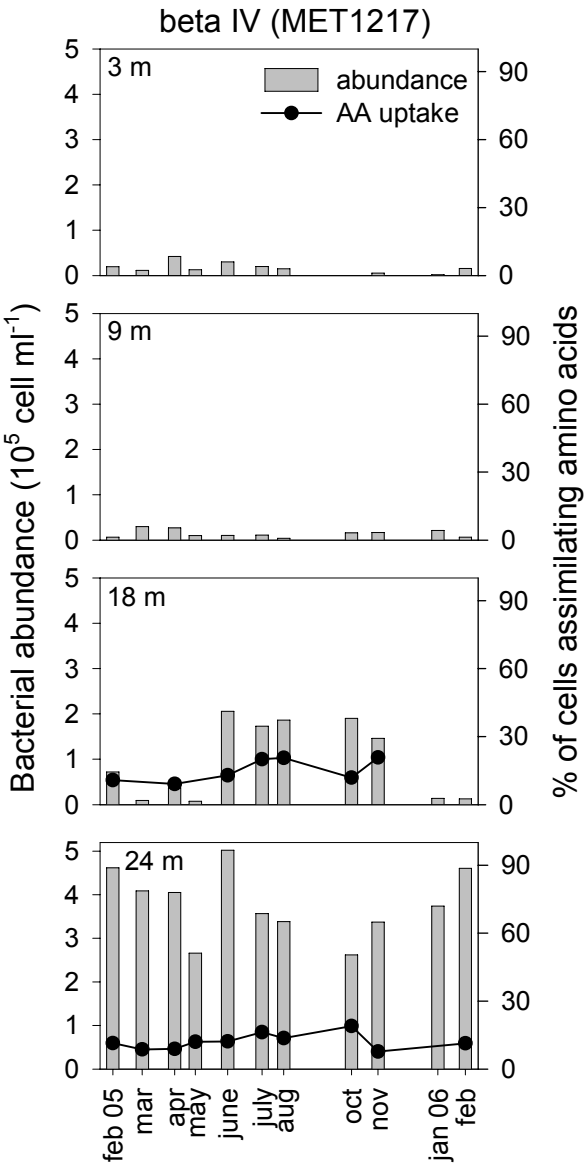


Salcher et al., Figure 3



Salcher et al., Figure 4



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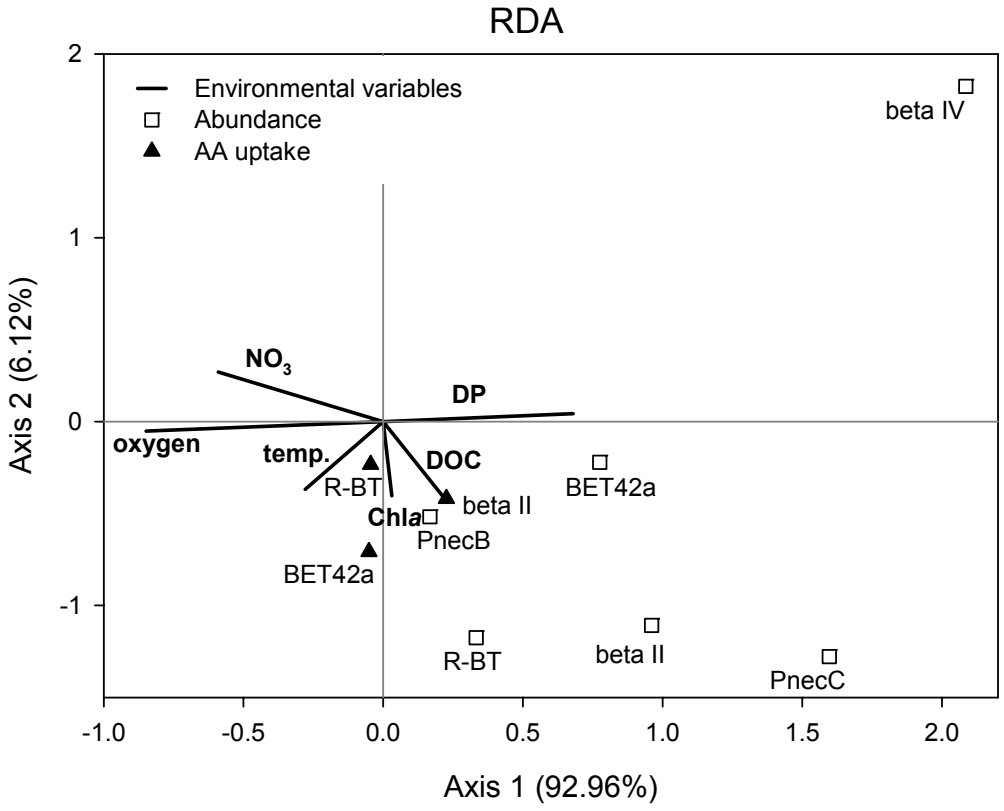
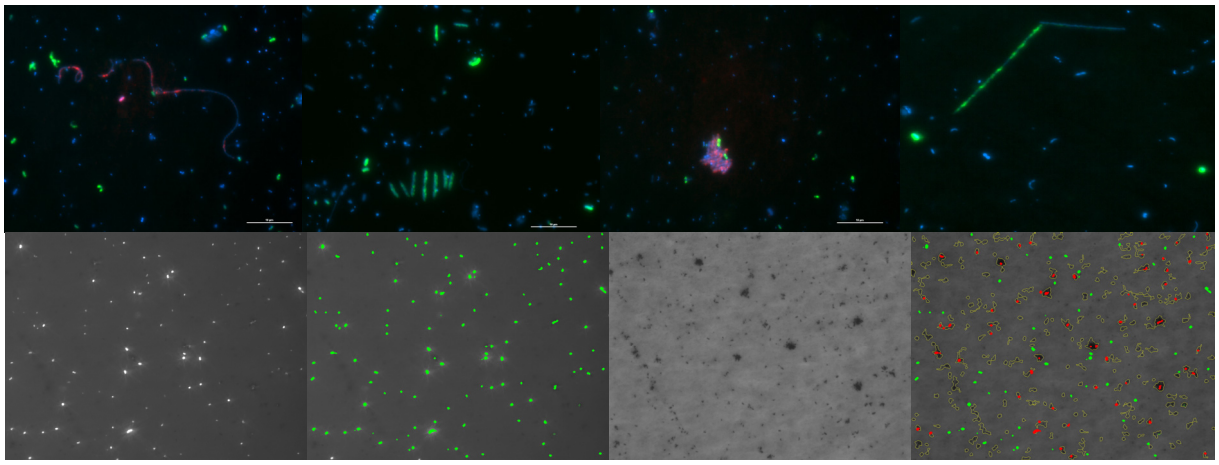


Salcher et al., Figure 6



manuscript VI

Abundance, biomass, or activity – which parameter mirrors best the standing stock of freshwater bacteria?

Salcher MM, Pernthaler J, Psenner R, Posch T

(still in prep…)

Abundance, biomass, or activity – which characteristic mirrors best the standing stock of freshwater bacterial clades?

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ABSTRACT

Three major bacterial lineages (*Betaproteobacteria*, *Actinobacteria*, and *Cytophaga-Flavobacteria*) are regarded as seemingly dominant in freshwater lakes. However, this view is mainly based on the determination of abundances via fluorescence *in situ* hybridization techniques (FISH) or on bacterial diversity estimated from clone libraries and fingerprinting techniques. The presented study elucidates, that the numerical importance of a bacterial lineage must not *per se* mirror its contribution to total bacterial biomass or activity in the oligo-mesotrophic lake Piburger See (Tyrol, Austria). Additionally, patterns of lineage dominance varied within the lake due to spatial and seasonal dynamics during a one year survey. Although Actinobacteria (ACT) numerically dominated the oxic epilimnion, less abundant lineages as Betaproteobacteria (BET) and Cytophaga-Flavobacteria (CF) contributed more to total bacterial biomass. In the sub- to anoxic hypolimnion, rare CF represented most bacterial biomass, followed by highly abundant BET. The contribution of ACT to total biomass was negligible in these water strata, although they still formed the numerical dominant fraction. The dominance of CF could have been even stronger, as results from clone libraries indicated an insufficient detection of *Bacteroidetes* by FISH with the oligonucleotide probe CF319a. Cells incorporating radiolabeled amino acids could be mainly attributed to ACT and BET. CF showed practically no affinity to the offered substrate, thus CF probably preferred high-molecular weight compounds also in our studied system. There is need to analyze not only the abundance of freshwater clades, but also their biomass and their affinity to various substrates to get a comprehensive picture of their ecological importance.

INTRODUCTION

Phylogenetic analyses of freshwater bacterioplankton (e.g. by fingerprinting methods or the establishment of clone libraries) usually result in sequences affiliated with a broad spectrum of bacterial phyla (Glöckner et al. 2000, Zwart et al. 2002) but gives only limited information about their numerical importance in the habitat. Through the application of fluorescence *in situ* hybridization (FISH) techniques, evidence is increasing that probably three bacterial lineages are prominent in freshwater systems (Amann et al. 1995, Alfreider et al. 1996, Bouvier & del Giorgio 2003). *Betaproteobacteria* (BET) were first identified as the dominant members of lakes and rivers (Glöckner et al. 1999). A further improvement of FISH via catalyzed reporter deposition (CARD-FISH) revealed the numerical importance of gram positive *Actinobacteria* (ACT), originally described for high mountain lakes, where ACT even exceeded numbers of BET (Glöckner et al. 2000, Sekar et al. 2003, Warnecke et al. 2005). As a third, *Cytophaga-Flavobacteria* (CF) of the phylum *Bacteroidetes* were found to be abundant in aggregates and deeper and/or anoxic water strata (Glöckner et al. 1999, Casamayor et al. 2000, Wu et al. 2006). The occurrence of other *Bacteria* (*Alphaproteobacteria*, *Gammaproteobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Firmicutes*) and *Archaea* has been proven for several lakes, however they seem to play a numerically minor role in most freshwater ecosystems.

Although the three apparently major lineages in lakes have been identified, it is not well understood which phyla can be dominant within a freshwater system, and what accounts for its prominent role. Until now, studies applying FISH were mainly focused on cell numbers within clades, mostly expressed as percentages of DAPI-positive particles, and theses values were regarded as

indicators for the dominance of bacterial lineages (Bouvier & del Giorgio 2003, Wu et al. 2006). However, cell numbers can not be the exclusive unit to characterize the importance of microorganisms in a lake. Bacterial abundances are needed to e.g. judge encounter rates and interactions with grazers (Jürgens & Matz 2002), but numbers are also an essential basis for the determination of bacterial biomass, which is the prerequisite for any modeling of fluxes within a microbial food web. Thus, when speaking about dominant bacterial lineages we should also pay attention to bacterial dimensions (morphotypes) to estimate their element content (Posch et al. 2007). Moreover, as bacteria can incorporate up to 50% of carbon originally bound by primary producers (Azam et al. 1983), they play a key role in the mineralization of dissolved organic material. Bacterial resource uptake was often studied via bulk activity measurements, such as the incorporation of radiolabeled substrates (Kirchman et al. 1985). The combination of micro-autoradiography methods with hybridization techniques (MAR-FISH, Lee et al. 1999) now allows for a closer look at resource uptake preferences and kinetics of bacterial lineages - a third aspect of dominance within freshwater bacteria.

We studied the bacterioplankton assemblage in an oligo-mesotrophic lake (Piburger See, Austria) for one year in terms of standing stocks of abundance, biomass, and cells with active amino acid incorporation. We hypothesized that (1) numerically dominant bacteria do not *per se* resemble the dominant biomass fraction or the majority of bacteria incorporating amino acids; (2) even less abundant bacteria can substantially contribute to total bacterial biomass or total activity. As our analyses of bacterial phyla were mainly based on CARD-FISH preparations, additionally, we created PCR-based clone libraries to prove the taxonomic resolution of our applied oligonucleotide probes.

MATERIALS AND METHODS

Study site and sampling

Piburger See is an oligo-mesotrophic, dimictic and holomictic lake in the Tyrolean Alps, Austria, at 913 m a.s.l. The maximum depth is 24.6 m, and nearly the entire hypolimnion gets anoxic during summer stratification. The lake's past was shaped by its eutrophication and re-oligotrophication (for further limnological details Tolotti & Thies 2002 and references therein). Water samples were taken monthly during one year (February 2005 to 2006) from 3, 9, 18, and 24 m depth. Forty ml samples were fixed with formaldehyde (2% final concentration) or freshly prepared buffered paraformaldehyde (pH 7.4, 2% final concentration) for determining bacterial abundance and CARD-FISH, respectively. Unfixed water samples (1 l) for MAR-FISH analyses and for the construction of 16S rRNA gene clone libraries were delivered to the lab at *in situ* temperatures within 2 hours. All samples were stored at 4°C until further processing.

Abundance of total bacteria and cyanobacteria

Two to 2.5 ml of formaldehyde-fixed samples were stained with DAPI (4',6-diamidino-2-phenylindole, 7 µg ml^{-1} final concentration, Porter & Feig 1980), filtered onto black polycarbonate filters (Osmonics, 0.22 µm pore size, 25 mm diameter), and evaluated with an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss, Germany). At least 1000 bacteria (UV excitation, Zeiss filter set 01) and 400 cyanobacteria (green excitation, Zeiss filter set 15) were counted per sample at a magnification of 1600x.

Abundance of bacterial clades

Ten to 15 ml of paraformaldehyde-fixed samples were filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2 µm pore size, 47 mm diameter), and stored at -20°C until further processing. CARD-FISH was carried out as previously described (Sekar et al. 2003) with the following oligonucleotide probes: EUBI-III for all *Bacteria* (Daims et al. 1999), BET42a for *Betaproteobacteria* (BET), HGC69a for *Actinobacteria* (ACT), CF319a for *Cytophaga-Flavobacteria* (CF), ALF968 for *Alphaproteobacteria*, and GAM42a for *Gammaproteobacteria* (Amann et al. 1995). Filter sections were counterstained with DAPI (1 µg ml^{-1}) and inspected with a Zeiss Axiophot microscope at a magnification of 1250x. At least 500 to 1000 DAPI-stained cells were counted, depending on the frequency of hybridized cells. Alternatively, a fully automated microscope (Zeiss AxioImager.Z1) was used for the evaluation of probe-positive bacteria (M. Zeder, unpublished, following the approach of Pernthaler et al. 2003).

Biomass of bacterial clades

For bacterial cell size determination we took two images from the same field of view, one at UV-excitation for the detection of all DAPI-positive cells (Channel 1) and a second at blue excitation for the detection of hybridized bacteria within the same microscopic field (Channel 2). Fifteen of these image pairs were recorded with a CCD camera from each filter section and processed with the image analysis program LUCIA D (Laboratory Imaging, Prague). First, we measured all morphotypes of channel 1 (DAPI-positive cells). Images of both channels 1 & 2 were combined by an object-based 'AND' function, thus selecting those DAPI-positive cells in channel 1, which had a counterpart also in channel 2 (hybridized bacteria). Thereafter, cell sizes and morphometric parameters were measured from channel 1 again (Posch et al. submitted). Cell size of bacteria was converted to biomass via the volume to carbon conversion factor of Loferer-Krössbacher et al. (1998).

Bacterial activity (MAR-FISH)

Unfixed water samples (5 ml) were incubated with [^{3}H]-amino acids (5 nM final concentration, 48 Ci $mmol^{-1}$ specific activity, Amersham) for 1 hour at *in situ* temperatures and fixed with paraformaldehyde (pH 7.4, 2% final concentration). After filtration (duplicates of 2 ml) onto white polycarbonate filters (Millipore, type GTTP, 0.2 µm pore size, 25 mm diameter) and CARD-FISH (see above), microautoradiography was carried out as following (Alonso & Pernthaler 2005): Filter sections were glued onto glass slides (2% Agarose, Seakem) and dipped into melted autoradiography emulsion (NTB emulsion, Kodak) in the darkroom, placed on ice for 10 min, and exposed in the dark at 4°C for 2-4 days. Thereafter, slides were developed with Dektol developer (Kodak) and Kodak fixer (Kodak) following the manufacturer's instructions. After drying, filters were embedded in a mounting medium containing DAPI (1 µg ml^{-1}). The evaluation of MAR-FISH filters was carried out with a fully automated microscope (Zeiss AxioImager.Z1) and an image analysis system (AxioVision 4.6, Carl Zeiss; M. Zeder, unpublished, following the strategy outlined in Alonso & Pernthaler 2005). No samples for MAR-FISH were collected in January 2006.

Environmental 16S rRNA clone libraries

Filter PCR of unfixed water samples form different depth and dates was done as previously described (Kirchman et al. 2001) with the primers GM3F and GM4R (Muyzer et al. 1995). After

purification of PCR products with the QIAquick PCR purification kit (QIAGEN), products were inserted into TOPO vectors (TOPO TA cloning kit for sequencing; Invitrogen) and cloned into competent *E. coli* cells according to the manufacturer's instructions. After screening of clones grown overnight on Luria-Bertani (LB) agar plates, plasmid preparations were done with the Montage™ Plasmid Miniprep96 Kit (Millipore) following the manufacturer's instructions. The sequencing reaction was accomplished with the ABI BigDye chemistry with the M13F vector primer and sequencing was done with an ABI 3130x Genetic Analyzer (Applied Biosystems). The obtained partial sequences were analyzed with the BLAST queuing system (www.ncbi.nlm.nih.gov/BLAST) for their phylogenetic affiliation. Moreover, nearly full-length sequences of BET were produced as described in Salcher et al. (submitted). All sequences affiliated with *Bacteroidetes* were subsequently checked for mismatches with probe CF319a (Manz et al. 1996) with the ARB software package (Ludwig et al 2004) using the PROBE_MATCH tool. As not all partial sequences had the base position 319, the closest full-length relatives were used instead.

Nucleotide sequence accession numbers

All 16S rRNA gene sequences have been deposited in EMBL under the accession numbers AM849424 to AM84946 and AM887995 to AM888176.

Statistical analyses

One way ANOVAs with pairwise *post hoc* comparisons (Scheffé method) were carried out to test for significant differences in cell numbers, biomass, and numbers of cells with active amino acid incorporation between oxic and sub- to anoxic samples, the studied phyla, calculated abundance-biomass-activity ratios, and differences between clone libraries and microscopy data. All raw data were arcsine (percentage data) or log(x+1) (cell numbers and biomasses) transformed prior to analysis.

RESULTS

Abundance, biomass and amino acid uptake of three bacterial phyla in relation to DAPI stainable particles

Bulk abundance, biomass, and numbers of cells with amino acid uptake were clearly correlated to the depth dependent oxygen gradient (Fig. 1). In the mostly anoxic hypolimnion we detected significantly more cells (factor 1.8), higher biomass (factor 2.4), and a higher number of active cells (factor 1.7) than in the epilimnion (one way ANOVA, $p < 0.001$ for all analyses). On average, 17.1 ± 6.7% of all DAPI stainable particles showed visible amino acid incorporation.

On average, only 52 ± 1.1% of all DAPI-stained particles were identified as *Bacteria* with the universal probe EUB I-III, and this proportion slightly rose with depth (Figs. 1, 2). In contrast, hybridized *Bacteria* constituted over 80% of DAPI stainable biomass, with higher values in the deeper strata than in the epilimnion. More than 80% of DAPI-stained cells with visible amino acid incorporation were also identified as *Bacteria* in the oxic water layers, whereas in the hypolimnion numbers of active *Bacteria* were equal to active DAPI-stainable particles (one way ANOVA, not significant).

Abundances of ACT, BET, and CF gave in sum more than 80% of all *Bacteria*. In the epilimnion the assemblage was dominated by ACT, followed by BET and low numbers of CF (Fig. 2).

In the oxygen-depleted water layers ACT and BET were equally abundant, again followed by CF. Hybridizations with probes specific for *Alphaproteobacteria* (ALF968) and *Gammaproteobacteria* (GAM42a) resulted in marginal numbers (on average 2.4% and 1.4% of EUB, respectively). Therefore it remains still unclear which other bacterial phyla contributed to *Bacteria* in Piburger See.

Approximately equal biomasses of the three studied phyla were found in the epilimnion of the lake (4.1, 5.3, and 5.1 µg C l^{-1}, for ACT, BET, and CF respectively). In the sub- to anoxic hypolimnion BET and CF had significantly higher biomasses (22.6 and 30.7 µg C l^{-1}, respectively) than ACT (6.4 µg C l^{-1}). In contrast to the numerical dominance, ACT accounted for the smallest part of bacterial biomass (Fig. 2), mainly due to their consistently small (<0.65 µm) morphologies (minute rods and vibrio-shaped cells). Eleven % of CF cells were filaments with extraordinary high biovolumes and the remaining CF were large (>0.65 µm) rods and cocci (data not shown). Thus, bacteria of this phylum dominated bacterial biomass in the anoxic zone and equaled ACT and BET-biomass values in the epilimnion. Within BET we found the highest morphological diversity, ranging from small (<0.5 µm) cocci to enlarged rods (data not shown). Similar to the numerical composition, a distinct fraction of *Bacteria*-biomass could not be attributed to any of the studied phyla, especially in the oxic zone of the lake (33.9%).

Bacteria showing amino acids uptake were dominated by ACT and BET, while almost no CF incorporated the offered substrate (Fig. 2). Whereas ACT constituted most of the active cells in the oxic zone, BET were highly active in the hypolimnion.

Abundance-, biomass-, and activity-ratios of ACT, BET, and CF

Although pronounced differences between the occurrence of bacteria for the studied phyla in the oxic and sub- to anoxic water column were detected (Fig. 2), we pooled all data for the presentation of parameter ratios (Table 1). EUB contributed significantly more to total biomass and to cells incorporating amino acids than to abundances of all DAPI stainable bacteria (Fig. 3, Table 1). This relationship was not found for the ratio of activity versus biomass. The biomass to abundance ratio of BET was not significant, but these bacteria were significantly more active than expected from their abundance. ACT had a low biomass compared to their numbers, but this phylum showed the highest activity to biomass ratio. CF had the highest biomass to abundance ratio but showed significantly lower activity than predicted from their abundance and biomass. Moreover, all ratios of BET were indistinguishable from those of EUB, whereas ratios of ACT and CF significantly differed from all other phyla.

Clone library versus CARD-FISH analysis (Fig. 3, Table S1, supplementary material)

The establishment of four clone libraries originating from different sampling depths in spring resulted in many sequences related to the three investigated bacterial phyla BET, ACT, and CF (75-82% for epilimnetic samples, 48% for the hypolimnetic sample). The clone library for the 24 m sample resulted in another 48% (29 clones) affiliated with the gamma I group of freshwater *Gammaproteobacteria*, although CARD-FISH with the probe GAM42a showed only a value of 0.3% of probe-positive bacteria for the same sample. Microscopic counting of autofluorescent *Cyanobacteria* also pointed to an overestimation by the PCR-based clone libraries (Fig. 3). The quantification of *Alphaproteobacteria* (probe ALF968) by CARD-FISH revealed equal results for both methods.

Sequences not covered by our applied probes were found within *Chloroflexi* (5 clones), *Deltaproteobacteria* (3 clones), *Firmicutes* (2 clones), and *Planctomycetes* (1 clone) (Table S1, supplementary material).

BET were underrepresented in clone libraries (Fig. 3), as well as ACT, which were detected in more than double proportions by CARD-FISH (one-way ANOVA: $F = 12.48$, $p = 0.012$, $n = 4$). The opposite was true for CF, which were significantly overestimated by PCR or underestimated by FISH ($F = 26.35$, $p = 0.002$, $n = 4$). A close look at CF-related sequences made clear that a large fraction of them showed one or several mismatches with the general probe CF319a (Manz et al. 1996). Only slightly significant differences were found between clones detectable with probe CF319a and microscopic counts with this probe ($F = 7.25$, $p = 0.036$, $n = 4$; Fig. 3).

DISCUSSION

Which bacterial phyla can dominate a freshwater lake? This study showed that applying a single methodological approach would have been insufficient to get a satisfying answer.

Abundance as indicator for dominant bacterial clades?

In general, numbers of *Bacteria* hybridizable with probe EUB I-III seem to be significantly lower than numbers of DAPI-stainable particles in the same freshwater samples (Bouvier & del Giorgio 2003, Wu et al. 2006). There are first indications that non-hybridizable but DAPI-positive particles represent the smallest morphologies within a sample (Posch et al. submitted). It is still unknown if these minute DAPI-stained particles are dead bacteria or bacteria at all (e.g., viruses or debris; Bettarel et al. 2000). In our study, no significant difference between all active (i.e. amino acids incorporating) DAPI-stained and EUB I-III positive cells were found in the sub- to anoxic hypolimnion, which proved that non-hybridizable but DAPI-positive particles were in fact not “important” members of the bacterioplankton in this environment. However, in the oxic layer of the lake 20% of DAPI stainable particles with active amino acid incorporation could not be attributed to EUB. It remains unknown if this discrepancy hints to a highly active lineage within *Bacteria* undetected by probe EUB I-III or to methodological problems concerning MAR-FISH.

Cumulative abundances of bacterial subgroups seldom reach the number of *Bacteria* detected with probe EUB I-III. Also in Piburger See, on average 17 % of *Bacteria* could not be attributed to any of the studied lineages by applying FISH. Results of our clone libraries implied a numerical underestimation of *Bacteroidetes* by hybridization with probe CF319a. This general probe covers only 38% of all published nearly full length sequences affiliated with the phylum *Bacteroidetes* (RDPII probe match, http://rdp.cme.msu.edu). Nevertheless for Piburger See, the application of probe CFB560 (O’Sullivan et al. 2002) that has theoretically a better coverage resulted in no detection at all (data not shown).

Were clone libraries also better indicators for the dominance of bacteria from different phyla in Piburger See than numbers gained by CARD-FISH? Although common patterns were detected for some phyla (BET, ALF), altogether the two methodological approaches did not reach a consensus. Discrepancies between PCR-based clone libraries and CARD-FISH could possibly be explained by the per cell number of genes coding for 16S rRNA (Farrelly et al. 1995). The amount of *rrn* operons per

cell can vary between 1 and 14, even for closely related species (Farrelly et al. 1995). Thus, some sequence types, probably overestimated by PCR (i.e. CF, *Gammaproteobacteria*, *Cyanobacteria*), could have contained more copies of *rrn* operons than e.g. ACT. However, this would be in contrast to marine systems where CF were found to be underrepresented in clone libraries (Cottrell & Kirchman 2000b). The small cell sizes of ACT indicate a generally low DNA content, whereas CF and also *Cyanobacteria* (data not shown) were represented by very large cells (Figs. 1, 2). Moreover, biases could be introduced during filter PCR due to gram-positive cell walls of ACT (Frostegard et al. 1999) and their high G+C content could cause lower amplification efficiency (von Wintzingerode et al. 1997).

Biomass is a valuable indicator for dominant bacteria

In contrast to abundance, the unit biomass allows for a direct comparison of energy and elements bound by different compartments within a trophic cascade. As recently shown, selective grazing by protists can affect the biomass of bacterial clades to a much higher degree than their abundance (Posch et al. 2007). BET and CF can be regarded as the major prey of bacterivorous flagellates in freshwater lakes (Jezbera et al. 2006), and their biomass was strongly reduced in the oxic zone of Piburger See throughout the year. There, numbers of flagellates were 2.3 times higher than in the sub- to anoxic zone (data not shown), a possible indication for grazing pressure in these water strata. Nevertheless, the biomass contribution of bacteria from these two clades was still equal to the biomass of ACT, which clearly dominated the assemblage in terms of cell numbers. Although ACT were abundant, their total biomass was generally low due to their generally minute cell sizes (e.g. Glöckner et al. 2000, Hahn et al. 2003, Posch et al. 2007). However, one third (33.9%) of the biomass hybridized with probe EUB I-III could not be attributed to any of the studied lineages in the epilimnion. This was mainly due to the occurrence of large filaments in spring, which could not be detected by any of the applied probes but EUB I-III. A second peak of filamentous bacteria in autumn in 24 m depth could be attributed to CF. Therefore, CF contributed most to bacterial biomass in anoxic samples, although present in low numbers. Bacteria of this lineage are known to form large cells and filaments (Alfreider et al. 1996, Pernthaler et al. 2004, Schauer & Hahn 2005, Šimek et al. 2007). The ratios of abundance versus biomass (Table 1, Fig. 2) probably mirror different ecological strategies developed by microbes affiliated to even such large phylogenetic units like ACT, BET, and CF. Nevertheless, it is of interest that bacteria of distinct lineages (e.g. CF) can strongly contribute to total bacterial biomass, even when their low abundances would classify them as less important.

Specific activities as indicator for dominant bacterial phyla?

ACT had the highest activity to biomass ratio, i.e. this lineage represented the most active biomass, here defined as amino acid incorporation. Therefore, our results suggest that bulk activity measurements simply do not provide quantitative data about bacterial protein and biomass production. A higher activity of ACT than of all *Bacteria* (EUB positive) in the assimilation of thymidine was detected in a drinking water reservoir (Nielsen et al. 2006), whereas similar DNA de novo synthesis rates for ACT and all *Bacteria* were reported for several high mountain lakes (Warnecke et al. 2005). Thus, these microbes may play a very important role in the remineralization of low molecular weight compounds of the organic carbon pool.

BET clearly dominated the fraction of active cells in the sub- to anoxic water strata. Within this phylogenetic lineage, especially the R-BT subcluster of beta I represented highly active bacteria (Horňák et al. 2006, Pérez & Sommaruga 2007, Salcher et al. submitted). BET affiliating to *Polynucleobacter* sp. (beta II) were also found to incorporate amino acids in high amounts, whereas beta IV bacteria did not incorporate these substrates (Salcher et al. submitted), as they presumably. All three phylotypes were present in Piburger See, occupying distinct ecological niches within the lake, which could explain the observed variability within total betaproteobacterial activity (Salcher et al. submitted). The low activity patterns found for CF correspond to the findings of Cottrell & Kirchman (2000a), that marine and estuarine CF were underrepresented in the proportion of the assemblage consuming amino acids. Low leucine uptake rates by freshwater CF were also described by several authors (Horňák et al. 2006, Pérez & Sommaruga 2006). Marine CF seem to prefer high-molecular weight compounds of the dissolved organic matter pool such as proteins or chitin (Cottrell & Kirchman 2000a) or different compounds of low molecular weight such as ATP (Alonso-Sáez & Gasol 2005). Therefore, our observation of low amino acids uptake by CF will not generally indicate a low activity of this lineage, as the choice of different substrates could have resulted in opposing findings.

Moreover, it should be taken in account that not all of the offered substrate may be used for bacterial biomass production, i.e. amino acids could also be respired for energy supply. Around one third of the gross uptake of ^{14}C amino acids was found to be respired by bacteria in Lake Constance with high seasonal variations (Weiss & Simon 1999), whereas higher respiration rates of ^{3}H labeled amino acids were detected in the oligotrophic Sargasso Sea (60 - 80%, Suttle et al. 1991) and coastal marine waters (20 - 60%, Carlucci et al. 1984).

Which bacterial clades can dominate a freshwater lake?

Our study shows that even very broad phylogenetic lineages may distinctly differ in their contribution to different ecological units such as numbers, biomasses, and amino acid incorporation. However, future research is needed to characterize the kinetics and affinities of bacteria from different lineages for various radiolabeled substrates in freshwater lakes, thus to characterize the competitive abilities of bacteria for available resources.

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Table 1: Biomass:abundance, activity:abundance, and activity:biomass ratios and standard deviations (calculated with raw % data) of all *Bacteria* (EUB), BET, ACT, and CF. Significant differences (one-way ANOVA with Scheffe post-hoc calculation) of raw data (in % of EUB, except for EUB in % of DAPI) are indicated by asterisks (**p < 0.005, ***p < 0.001). Data were arcsine transformed prior to statistical analysis.

		Abundance	Biomass
EUB	Biomass	1.5 ± 0.2***	
	Activity	1.6 ± 0.6***	1.1 ± 0.2
BET	Biomass	1.2 ± 0.4	
	Activity	1.7 ± 0.6***	1.7 ± 0.8**
ACT	Biomass	0.4 ± 0.1***	
	Activity	1.1 ± 0.4	3.3 ±1.4***
CF	Biomass	3 ± 1.1***	
	Activity	0.3 ± 0.2***	0.1 ± 0.1***

Figure legends:

Figure 1: Abundances, numbers of cells with active amino acid incorporation, and biomass of DAPI stainable particles and *Bacteria*. Left panel: Samples from the oxic epi- and metalimnion; right panel: samples from the sub- to anoxic hypolimnion (< 1 mg oxygen l^{-1}). Abbreviations: DAPI: abundance of DAPI stainable particles; EUB: abundance of *Bacteria* hybridized with probe EUB I-III; DAPI MAR+: DAPI stainable particles with active amino acid incorporation; EUB MAR+: *Bacteria* with active amino acid incorporation. DAPI BIO: Biomass of DAPI stainable particles; EUB BIO: Biomass of *Bacteria.* Different letters above the bars indicate significant differences between the cell numbers (one way ANOVA with Scheffe *post hoc* comparisons, $p < 0.001$).

Figure 2: Left part of each graph: Contributions of BET, ACT, and CF to total abundances, biomass, and cells with amino acid incorporation (in % of DAPI and % of EUB). Right part of each graph: Abundances (10^5 cells ml^{-1}), biomasses (µg C l^{-1}), and abundances of active cells (10^5 cells ml^{-1}) of BET, ACT, CF, and other EUB. Left panels: Samples from the oxic epi- and metalimnion; right panels: samples from the sub- to anoxic hypolimnion (< 1 mg oxygen l^{-1}). Abbreviations: ACT = *Actinobacteria*, BET = *Betaproteobacteria*, CF = *Cytophaga-Flavobacteria*, EUB = *Bacteria*. Different letters above the bars indicate significant differences between the studied lineages (one way ANOVA with Scheffe *post hoc* comparisons, $p < 0.05$).

Figure 3: Left panels: Abundance, biomass, and activity contributions of *Bacteria* (EUB) to total DAPI-stained particles. Right panels: Abundance, biomass, and activity contributions of BET, ACT, and CF to total hybridized *Bacteria*. For abbreviations see legend of figure 2. Empty symbols: oxic samples; symbols with x-hair: sub- to anoxic samples.

Figure 4: Bacterial assemblage composition in Piburger See gained via sequencing of clone libraries (CL; % of clones) in comparison to a microscopic evaluation of FISH preparations (M; % of EUB). For abbreviations see legend of figure 2.

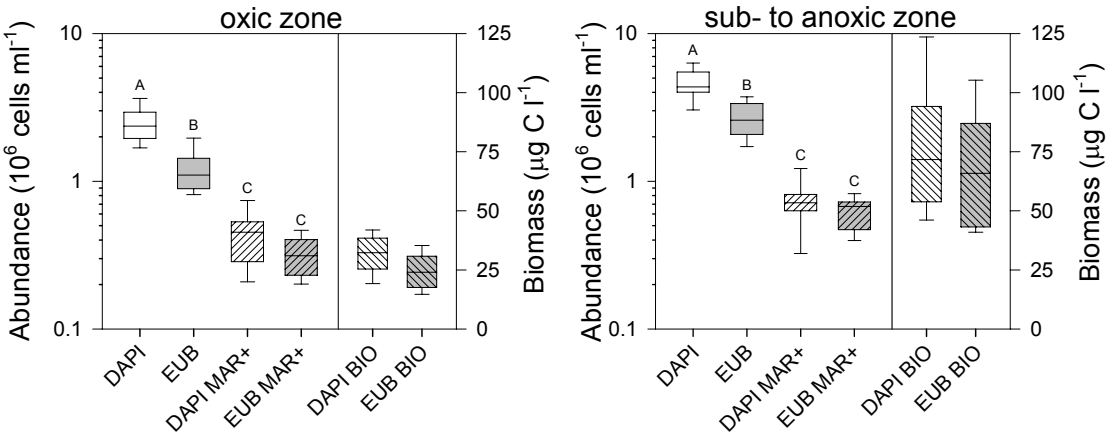

Figure 1, Salcher et al.

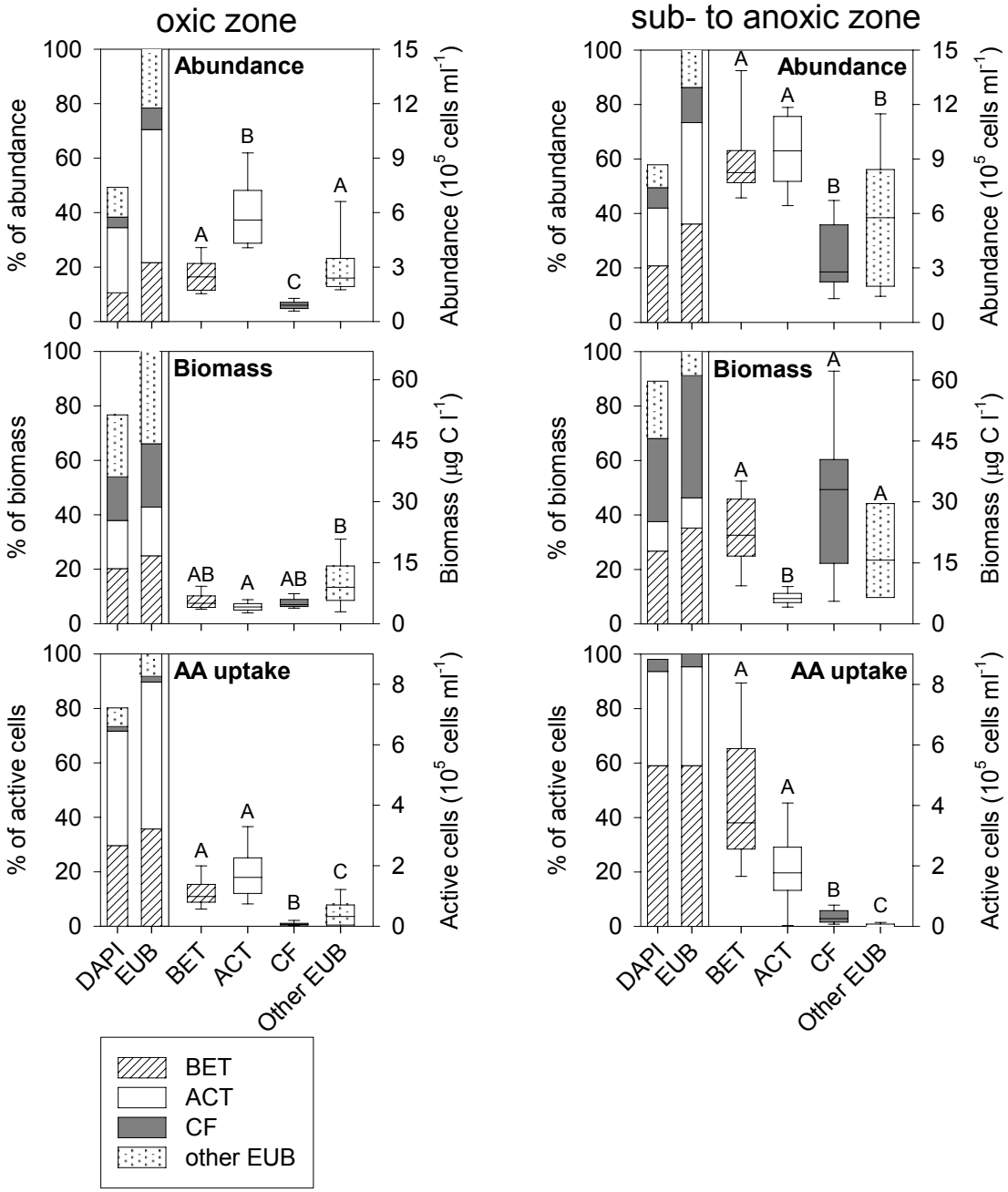

Figure 2, Salcher et al.

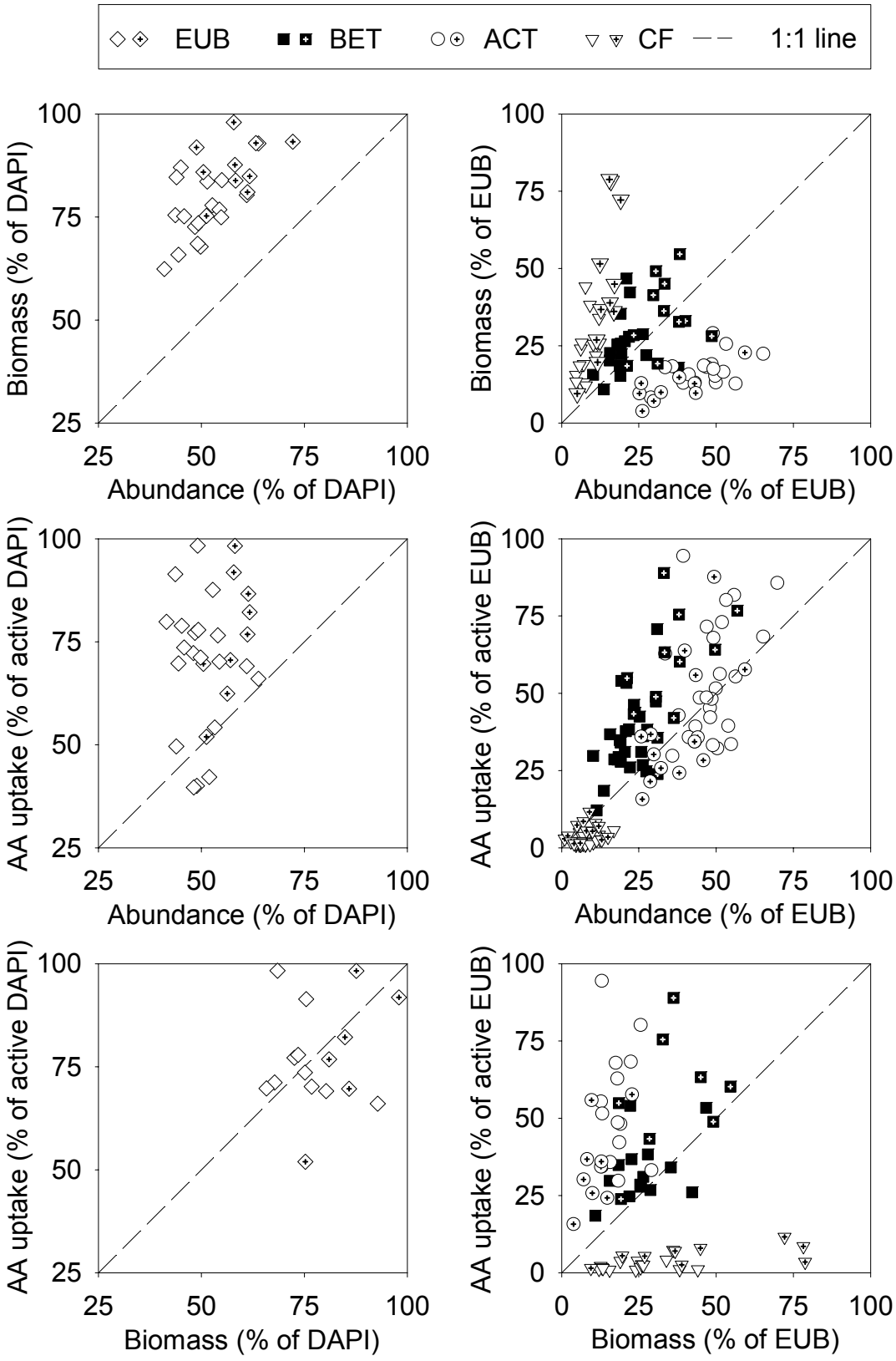

Figure 3, Salcher et al.

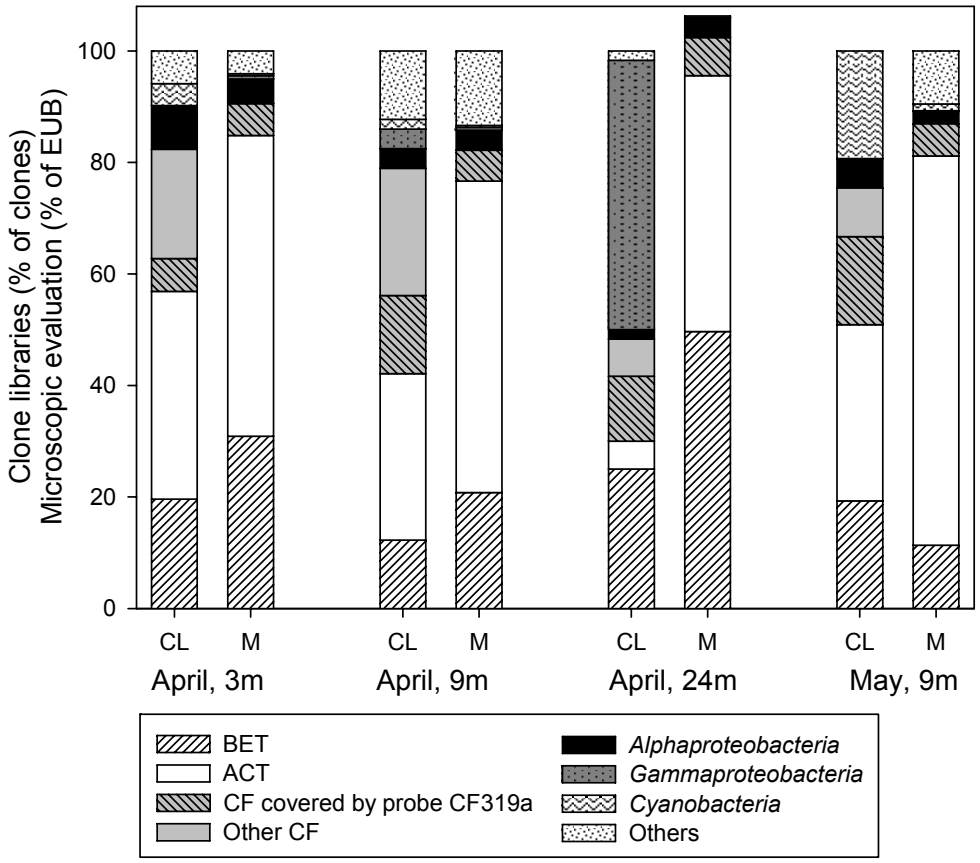

Figure 4, Salcher et al.

Supplementary Material:
Table S1: Phylogenetic affiliation of 16S rRNA gene sequences derived from four clone libraries. *Betaproteobacteria* are excluded (see Salcher et al. (submitted) for a phylogenetic tree of betaproteobacterial sequences). Freshwater clusters were classified according to Glöckner et al. (2000). A + or – in the line of CF319a indicates the detection (+) of sequences by probe CF319a, or the occurrence of one or several mismatches (-). An asterisk indicates no base position 319 in the particular sequence.

Clone	Sample depth (m)	Closest relative	Accession number	Similarity (%)	Freshwater cluster	CF319a
Alpha-Proteobacteria						
PIB-1	9	clone HTH6	AF418965	99	alpha V	
PIB-2	24	clone TH1-19	AM690823	99	alpha V	
PIB-3 – 9	3, 9	clone ST5-4	DQ501352	99–100	alpha V	
PIB-10	3	clone TW11-2	DQ501359	99	alpha V	
Gamma-Proteobacteria						
PIB-58	9	clone: SRRB72	AB240527	96	gamma I	
PIB-59 – 66	9	*Methylobacter psychrophilus*	AF152597	99	gamma I	
PIB-67 – 78	3, 24	bacterium FukuN13	AJ290055	96–99	gamma I	
PIB-79	9, 24	clone 1H_73	AY546504	97	gamma I	
PIB-80 – 85	24	clone Pav-010	DQ642324	99–100	gamma I	
PIB-86 – 87	24	clone CbS1s.04	EF014669	100	gamma I	
PIB-88	24	clone: RB136	AB240311	96		
Delta-Proteobacteria						
PIB-89	9	bacterium FukuN97	AJ290002	98		
PIB-90	9	bacterium FukuN9	AJ290009	99		
PIB-91	3	clone Won5(0714)	DQ839501	98		
Actinobacteria						
PIB-92	9	clone:S9F-52	AB154310	100	acI-A	
PIB-93 – 97	3, 9	bacterium GKS2-103	AJ290024	99–100	acI-A	
PIB-98	9	clone 253	AJ536838	99	acI-A	
PIB-99	3	clone S7	AJ575508	99	acI-A	
PIB-100	3	clone N3	AJ575530	99	acI-A	
PIB-101	3	clone SL39	AY466489	99	acI-A	
PIB-102	9	clone LiUU-3-127	AY496980	99	acI-A	
PIB-103	9	clone LiUU-5-178	AY496985	99	acI-A	
PIB-104 – 105	3	bacterium P38.5	AY752087	99	acI-A	
PIB-106	3	clone PRD18G08	AY948060	100	acI-A	
PIB-107 – 108	3, 9	clone ML-3-12.2	DQ520158	99	acI-A	
PIB-109	9	clone S8	AJ575509	99	acI-B	
PIB-110	9	clone LiUU-3-254	AY496981	99	acI-B	
PIB-111 – 112	3	clone BL11-15	DQ316323	99–100	acI-B	
PIB-113 – 117	9	clone NM1	AJ575534	98–99	acI-C	
PIB-118	9	clone NM3	AJ575536	99	acI-C	
PIB-119	9	clone ST11-18	DQ316362	99	acIV	
PIB-120 – 122	3, 24	clone N2	AJ575529	99–100	acIV-A	
PIB-123	9	clone ST11-15	DQ316360	100	acIV-A	
PIB-124 – 126	3, 24	clone SOC1 8E	DQ628963	99–100	acIV-A	
PIB-127 – 134	9	clone S1	AJ575504	98–99	acIV-B	
PIB-135 – 143	3,9	clone NM2	AJ575535	99–100	acIV-B	
PIB-144	3	clone BL11-28	DQ316331	99	acIV-B	
PIB-145	3	clone: BD4-12	AB015562	100		
PIB-146	24	clone D2	AJ867923	100		
PIB-147	9	clone TH3-72	AM690963	99		
PIB-148	9	clone STH5-14	DQ316384	99		

Clone	Sample depth (m)	Closest relative	Accession number	Similarity (%)	Freshwater cluster	CF319a
Bacteroidetes						
PIB-149	3	bacterium SY6-50	AF296201	97	cfI	−
PIB-150 – 151	9	clone S155	AF507731	98	cfI	−*
PIB-152	9	clone LPU78	AF527600	96	cfI	−*
PIB-153	9	clone RSC-II-81	AJ252697	93	cfI	−
PIB-154	24	clone UH4	DQ298000	96	cfI	−*
PIB-155	9	clone TW11-23	DQ501362	99	cfI	−
PIB-156	3	bacterium SY3-10	AF107517	99	cfI	−
PIB-157	3	bacterium SY4-2	AF107525	100	cfI	−
PIB-158	3	clone TLM09/TLMdgge12a	AF534433	99	cfI	−*
PIB-159 – 166	3, 9, 24	clone FNE11-5	DQ501312	98–99	cfI	− (*)
PIB-167 – 170	3, 9	clone ADK-WSe02-05	EF520604	98–100	cfI	−*
PIB-171 – 172	9, 24	clone ES3-44	DQ463263	95–96	cfII	+
PIB-173 – 174	24	ADK-MOe02-29	EF520552	96–97	cfII	+
PIB-175 – 179	3, 9	clone TLM12	AF534436	96–99	cfII	+ (*)
PIB-180	9	bacterium CS910	AY124339	99	cfII	+*
PIB-181	24	clone LiUU-3-199	AY509279	97	cfII	+
PIB-182	9	clone ES3-12	DQ444136	96	cfII	+
PIB-183	9	clone ES3-36	DQ463255	96	cfII	+
PIB-184 – 186	3, 24	clone Chun-w-42	EF632772	97–99	cfII	+*
PIB-187 – 188	9	clone CR98-5-02	AF428732	99–100	cfIII	−*
PIB-189	24	clone NE02	AJ575726	100	cfIII	−*
PIB-190	9	clone TH3-69	AM690960	99	cfIII	−
PIB-191	3	clone 207ds20	AY212653	99	cfIII	−
PIB-192 – 194	9	clone PRD18G03	AY948057	99	cfIII	−*
PIB-195	3	clone ST5-10	DQ501343	99	cfIII	−
PIB-196	9	clone SOC1 4G	DQ628941	99	cfIII	−*
PIB-197	9	clone JEG.b12	DQ228391	97		+
PIB-198 – 199	3, 9	clone ST11-9	DQ501342	96–99		+*
PIB-200	9	clone 47	AF513095	99		+*
PIB-201 – 202	9	TLM11/TLMdgge04	AF534435	98		+*
PIB-203	9	clone LiUU-5-303	AY509378	99		+*
PIB-204 – 205	9, 24	clone SE168	DQ327682	96–97		+ (*)
PIB-206	9	clone ST11-20	DQ501331	96		+*
PIB-207	9	clone SILK35	EF467509	98		+*
Chloroflexi						
PIB-208	3	clone SL56	AY466492	99		
PIB-209 – 211	3, 9	clone LiUU-3-250	AY509486	98–99		
PIB-212	9	clone TW11-28	DQ501364	99		
Planktomycetes						
PIB-213	9	clone CRD99-30	AF428608	99		
Firmicutes						
PIB-214	9	clone P4-29	AF523328	100		
PIB-215	24	clone 965004D07.x1	DQ065372	98		
Cyanobacteria						
PIB-216 – 220	3, 9	clone LD4	AJ006279	98		
PIB-221 – 222	3, 9	clone FrE313	AY151736	96–97		
PIB-223 – 229	9	clone ELB16-090	DQ015808	98–99		

…appendix…

protocols

CARD–FISH PROTOCOL

fixation & filtration

- Fix x ml sample with x ml PFA (2% f.c.) ~1 h @ RT (or overnight @ 4°C)
- Filter x ml sample onto white polycarbonate-filters (0.2 µm pore size + supporting filter, 0.45 µm pore size), rinse with MQ, let filters air dry, store @ -20°C

embedding in agarose & enzyme pretreatment

- Melt 0.2 % agarose in microwave oven, let it cool down to ~40°C
- Clean a glass plate (or petri dish) with EtOH
- Dip filters in agarose, put them on glass plate or petri dish (bacteria facing up), let filters dry (@ 30-40°C for 10-20 min)
- Remove filters from the glass plate by pipetting EtOH onto them and peel them off carefully
- Air dry filters and store them @ -20°C (or go on with the protocol)
- Incubate filters in 0,01 M HCl for 10 min @ RT
- Wash filters in PBS and MQ (RT)
- Prepare fresh lysozyme solution
  100 mg lysozyme (10 mg/ml)
  1 ml 0,5 M EDTA (pH 8)
  1 ml 1 M Tris/HCl (pH 8)
  8 ml MQ
- Incubate filters in lysozyme for 60 min @ 37°C
- Wash filters in PBS and MQ (RT)
- Prepare fresh achromopeptidase solution
- 2 µl Achromopeptidase-Stock + 1 ml NaCl-Trispuffer
- Incubate filters in achromopeptidase for 30 min @ 37°C
- Wash filters in MQ and EtOH (RT), let them air dry and store @ -20°C (or go on with the protocol)

Alternatively to achromopeptidase, filters can also be pretreated with Proteinase K:

- Prepare fresh Proteinase K solution
  75 µl Proteinase K stock (1:100)
  1 ml Tris/HCl, pH 8
  0.5 ml EDTA, pH 8
  8.425 ml MQ
- Incubate filters in Proteinase K for 30 min @ 37°C
- Wash filters in MQ and EtOH (RT), let them air dry and store @ -20°C (or go on with the protocol)

hybridization & tyramide signal amplification

- Cut filters into pieces, label them with a pencil
- Mix 300 µl (900 µl) of hybridization buffer + 2 µl (6 µl) probe (0.5 or 1.5 ml tube, tube must be 2/3 filled with buffer), you can hybridize more filter sections in a Petri dish (up to ~50), but be careful that all sections are covered with buffer
- Hybridize filter sections for 2 h @ 35°C (or up to 24 h!)
- Prepare washing buffer
  500 µl 0,5 M EDTA(pH 8)
  1000 µl 1 M Tris/HCl (pH 7.4)
  x µl 5 M NaCl
  fill up to 50 ml with MQ
  50 µl SDS
- Preheat washing buffer in water bath @ 37°C
- Wash filters in washing buffer for 20-30 min @ 37°C
- Incubate filters in PBST for 45 min @ 37°C
- Prepare 0,15% H_2O_2
  1000 µl 1 x PBS (or MQ)
  5 µl 30% H_2O_2
- Mix 1 ml of amplification buffer + 10 µl 0.15% H_2O_2 + 2 µl fluorescently labelled tyramide
- Dab filters onto blotting paper to remove excess liquid but don't let filters run dry
- Incubate filters in tyramide solution for 30 min @ 37°C in the dark
- Dab filters onto blotting paper to remove excess liquid but don't let filters run dry
- Incubate filters in PBST for 15 min @ RT in the dark
- Wash filters in MQ and EtOH (@ RT in the dark)
- Let filters air dry (in the dark)
- Embed filters in DAPI-MIX or store them @ -20°C

Volumes of formamide and water for 20 ml of hybridization buffer, volumes of 5 M NaCl in 50 ml of washing buffer. The Na+ concentration is calculated for stringent washing at 37°C after hybridization at 35°C.

% formam. in hybridization buffer	ml formamide	ml water	ml of 5 M NaCl in washing buffer
20	4	10	1350
25	5	9	950
30	*6*	*8*	*640*
35	*7*	*7*	*420*
40	*8*	*6*	*270*
45	*9*	*5*	*160*
50	*10*	*4*	*90*
55	*11*	*3*	*30*
60	*12*	*2*	*0*
65	*13*	*1*	*0*
70	*14*	*0*	*0*

Formamide concentration of probes:

EUB I-III	55 %
BET42a	55 % competitor!
GAM42a	55 % competitor!
Bet2-870	55 %
MET1217	40 % competitor & helpers!
Acl-1214	55 %
PnecABD	55 %
PnecB	60 %
ALF968	55 %
CF319a	55 %
HGC69a	30-35 %
R-BT065	55 %
Acl-852	55 % helpers!
Acl-840-1,2,3	30 % competitors & helpers!
PnecC	55 %

double hybridization

After hybridization and CARD with the first probe filters can be stored at –20°C or immediately hybridized with the second probe. It is also possible to use filter sections that are already embedded in DAPI-MIX, you just have to remove the cover slip and wash filters in excessive EtOH and MQ to remove all oil.

- Incubate filter sections in 0.01 M HCl for 10 min @ RT
- Mix 300 µl (900 µl) of hybridization buffer + 2 µl (6 µl) of the second probe
- Hybridize filter sections for 2 h @ 35°C
- Prepare washing buffer
  500 µl 0,5 M EDTA (pH 8)
  1000 µl 1 M Tris/HCl (pH 7.4)
  x µl 5 M NaCl
  fill up to 50 ml with MQ
  50 µl SDS
- Preheat washing buffer in water bath @ 37°C
- Wash filters in washing buffer for 20-30 min @ 37°C
- Incubate filters in PBST for 45 min @ RT
- Prepare 0,15% H_2O_2
  1000 µl 1 x PBS (or MQ)
  5 µl 30% H_2O_2
- Mix 1 ml of amplification buffer + 10 µl 0.15% H_2O_2 + 2 µl fluorescently labelled tyramides. **Here, a second fluorochrome must be used,** e.g. Alexa488 for the first hybridization & Alexa546 for the second
- Dab filters onto blotting paper to remove excess liquid but don't let filters run dry
- Incubate filters in tyramide solution for 30 min @ 37°C in the dark
- Dab filters onto blotting paper to remove excess liquid but don't let filters run dry
- Incubate filters in PBST for 45 min @ RT in the dark
- Wash filters in MQ and EtOH (@ RT in the dark)
- Let filters air dry (in the dark)
- Embed filters in DAPI-MIX or store them @ -20°C

tyramide synthesis

TYR stock
1 ml dimethylformamide
10 µl triethylamine (15 ml Falcon tube)
10 mg Tyramine-HCl
(100 µl TYRstock = 5.76 µmol l^{-1})

Succinimidyl ester
1 mg active ester
100 µl dimethylformamide
(1 mg Alexa488 = 1.6 µmol l^{-1})
Esters are light sensitive and prone to hydrolysis. Therefore prepare shortly before tyramides synthesis! Cool esters on ice until they are used for synthesis.

Synthesis
- 100 µl Alexa488 + 25.2 µl TYR stock
- Incubate at RT in the dark for ~12 h
- Dilute to 1 ml with absolute Ethanol (874.8 µl for Alexa488)
- Make aliquots (~50 µl), store @ -20°C (stable for at least 1 year – in the fridge is also possible, but only for the aliquot you use)

Alternatively you can desiccate tyramides for longer storage
- Desiccate aliquots in a freeze dryer or under vacuum at room temperature. Desiccated tyramides are stable for years @ -20°C.
- For use, reconstitute desiccated tyramides with 50 µl MQ or DMF containing 20 mg ml^{-1} p-iodophenylboric acid (IPBA). IPBA will enhance the CARD-FISH signal! Caution: Alexa350-labeled tyramides should be dissolved in MQ and stored in the fridge; tyramides dissolved in DMF can be stored in the freezer.

Other fluorochromes:
100 µl Alexa546 + 14.7 µl TYR stock
100 µl Alexa633 + 13.1 µl TYR stock
500 µl Alexa350 + 193 µl TYR stock (5 mg active ester + 500 µl DMF)
10 ml Carbofluorescein + 3.3 ml µl TYR stock (100 mg ester + 10 ml DMF)

material

Filtration device
Falcon tubes (15 ml, 50 ml)
Eppendorf tubes (0.5 ml, 1.5 ml)
Petri dishes
Glass plate
Blotting paper
Slides & cover slips
Pipettes
Scalpel
Forceps
Hybridization oven & water bath

solutions

20% PFA: 100ml
Heat 90 ml MQ @ 60°C
Solve 20 g PFA (cover vessel)
It dissolves more quickly if you add some drops of NaOH
10 ml 10X PBS
Adjust pH to 7.2
Cool down to +4°C
Filter sterile
Store @ -20°C (or +4°C for several days)
Freshly prepared PFA is even better

0.1 % Agarose: 200 ml
0.2 g Agarose
200 ml MQ
Stir and heat until agarose is completely dissolved
Storage @ RT, melt it before use (microwave)

10 % Blocking reagent:
Prepare buffer
  0.15 M NaCl (0.438g)
  0.1 M maleic acid (0.5805g)
  50 ml MQ
  Adjust pH to 7.5
  Filter sterile
50 ml buffer
5 g Blocking reagent
Stir & heat until it is completely dissolved
Store @ -20°C or +4°C (several days)

Proteinase K solution
75 µl Proteinase K stock (1:100)
1 ml Tris-HCl, pH 8
0.5 ml EDTA, pH 8
8.425 ml MQ

Achromopeptidase stock (30 KU)
40 mg (100 KU)
3.3 ml MQ
Aliquote
Store @ -20°C

Achromopeptidase solution
2 µl Achromopeptidase stock
1 ml NaCl-Tris buffer
Prepare fresh

1 x PBS, pH 7.6: 1l
8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4

0.24 g KH_2PO_4
1000 ml MQ
Adjust pH to 7.6
Autoclave
Store @ RT

10 x PBS, pH 7.6:
Ten times more, for example 500ml:
40 g NaCl
1 g KCl
7.2 g Na_2HPO_4
1.2 g KH_2PO_4
500 ml MQ
Adjust pH to 7.6
Autoclave
Store @ RT

0.01% PBST, pH 7.6: 500 ml
500 ml 1 x PBS
50 µl Triton X-100
Store @ RT

Lysozyme solution: 10 ml
100 mg Lysozym (10 mg ml^{-1})
1 ml 0,5 M EDTA
1 ml 1 M Tris/HCl
8 ml MQ
Prepare fresh

NaCl-Tris buffer, pH 8: 50 ml
100 µl 5 M NaCl
500 µl 1 M Tris/HCl
Fill to 50 ml with MQ
Adjust pH to 8
Filter sterile
Store @ RT

5 M NaCl: 400 ml
116.884 g NaCl
400 ml MQ
Autoclave
Store @ RT

1 M Tris-HCl, pH 7.4 / pH 8: 100 ml
15.76 g Tris-HCl
100 ml MQ
Adjust pH to 7.6 or 8
Autoclave
Store @ RT

0.5 M EDTA, pH 8: 100 ml
14.612 g EDTA
100 ml MQ
Adjust pH to 8
Stir until it is completely dissolved (after pH adjustment it's more easy)
Autoclave
Store @ RT

10 % SDS: 50 ml
5 g SDS
50 ml MQ
Dissolve @ 40°C
Filter sterile
Store @ RT

0.15% H2O2:
1 ml 1 x PBS (or MQ)
5 µl 30% H_2O_2
Prepare fresh

DAPI-Mix, pH 9:
5 x Glycerol (e.g. Citiflour)
1 x Vectashield
1 x PBS
1 µg DAPI ml^{-1}
Adjust pH to 9
Store @ +4°C in the dark

Amplification buffer:
4 ml 10 x PBS
16 ml 5 M NaCl
4 g dextran sulfate
Stir & heat (~40°C) until it is completely dissolved
Cool down to +4°C
0.4 ml blocking reagent
Fill up to 40 ml with MQ
Store @ +4°C (several weeks)

Hybridization buffer (20 ml)
3600 µl 5 M NaCl
400 µl 1 M Tris/HCl (pH 7.4)
2 g dextran sulfate
Stir & heat (~40°C) until it is completely dissolved
Cool down to +4°C
x ml MQ (table X)
x ml formamide (table X)
2000 µl Blocking reagent
20 µl SDS
Aliquote
Store @ -20°C

Washing buffer
500 µl 0.5 M EDTA
1000 µl 1 M Tris/HCl
x µl NaCl (table X)
fill to 50 ml with MQ
50 µl SDS

ORDERING INFORMATION

Agarose
SeaKem LE agarose
BMA (BioWittaker Molekular Applications)
www.bmaproducts.com
50001 (25 g)

Triton X-100
Sigma-Aldrich Co www.sigmaaldrich.com
93426

SDS
Sigma-Aldrich Co www.sigmaaldrich.com
71725 (50 g)

EDTA
Sigma-Aldrich Co www.sigmaaldrich.com
03677

Blocking reagent
Roche Diagnostics GmbH
www.roche-applied-science.com
1096176 (50 g)

Maleic Acid
Sigma-Aldrich Co www.sigmaaldrich.com
63180 (100 g)

Dextran sulfate
Sigma-Aldrich Co www.sigmaaldrich.com
D8906 (100 g)

Formamide
Sigma-Aldrich Co www.sigmaaldrich.com
47671 (250 ml)

30% H2O2
Sigma-Aldrich Co www.sigmaaldrich.com
H1009 (5 ml)

Lysozym
Sigma-Aldrich Co www.sigmaaldrich.com
L7651 (10 g)

Achromopeptidase
Sigma-Aldrich Co www.sigmaaldrich.com
Enzyme commission Number (EC): 3.4.21.50
A3547 (100 KU)

HRP-labeled Probes
ThermoHybaid
www.thermohybaid.de

Paraformaldehyde
Sigma-Aldrich Co www.sigmaaldrich.com
76240 (250 ml)

Citifluor AF1
Linaris Biologische Produkte www.linaris.de
E6016BG (25 ml)

Vectashield
Linaris Biologische Produkte www.linaris.de,
H-1000 (10 ml)

DAPI
Sigma-Aldrich Co www.sigmaaldrich.com
D9542 (10 mg)

N,N-Dimethylformamide
Sigma-Aldrich Co www.sigmaaldrich.com
D4551 (250ml)

4-Iodophenylboronic acid
Sigma-Aldrich Co, www.sigmaaldrich.com
47, 193-3 [5122-99-6]

Triethylamine
Sigma-Aldrich Co www.sigmaaldrich.com
90335 (100 ml)

Tyramine HCl
Sigma-Aldrich Co www.sigmaaldrich.com
T2879-1gr

Fluorochromes
Invitrogen
www.invitrogen.com
e.g. Alexa 488 succinimidyl esther
A 20000 (1 mg)

Absolute Ethanol
Merck
www.merck.ch
100986 (1000 ml)

Polycarbonate filters
Millipore
www.millipore.com
White, 45 mm diameter, 0.2 µm pore size: # GTTP04700
White, 25 mm diameter, 0.2 µm pore size: # GTTP02500

Supporting filters
Sartorius AG
www.sartorius.com
47 mm diameter, 0.45 µm pore size:
11306-47-N
25 mm diameter, 0.45 µm pore size:
11306-25-N

Commonly used oligonucleotide probes targeting freshwater bacteria.

Probe	Specificity	Sequence (5' to 3')	Target site (rRNA, 5' position)	FA %	Reference
General probes					
EUB I-III	*Bacteria*	GCW GCC WCC CGT AGG WGT	16S, 338	55	Daims et al. 1999
ALF968	*Alphaproteobacteria*	GGT AAG GTT CTG CGC GTT	16S, 968	55	Neef 1997
BET42a	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	23S, 1033	55	Manz et al. 1992
BET42a-C	Competitor for BET42a	GCC TTC CCA CAT CGT TT	23S, 1027	55	Manz et al. 1992
GAM42a	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	23S, 1027	55	Manz et al. 1992
GAM42a-C	Competitor for GAM42a	GCC TTC CCA CTT CGT TT	23S, 1033	55	Manz et al. 1992
CF319a	*Cytophaga-Flavobacteria (Bacteroides)*	TGG TCC GTG TCT CAG TAC	16S, 319	55	Manz et al. 1996
HGC69a	*Actinobacteria*	TAT AGT TAC CAC CGC CGT	23S, 1907	30	Roller et al. 1994
Specific probes					
Acl-840-1	Subcluster of acl lineage of *Actinobacteria*	TCG CAC AAA CCG TGG AAG	16S, 840	30	Warnecke et al. 2005
Acl-840-2	Subcluster of acl lineage of *Actinobacteria*	TCG CAG AAA CCG TGG AAG	16S, 840	30	Warnecke et al. 2005
Acl-840-3	Subcluster of acl lineage of *Actinobacteria*	TCG CAC AGA CCG TGG AAG	16S, 840	30	Warnecke et al. 2005
Acl-840-H1	Helper 1 for Acl-840	CTA GYG CCC AYC GTT TAC GG	16S, 810	30	Glöckner et al. 2000
Acl-840-H2	Helper 2 for Acl-840	GTT CSC ACA ACT AGY GCC CA	16S, 820	30	Glöckner et al. 2000
Acl-840-H3	Helper 3 for Acl-840	GGG GCR CTT AAT GCG TTA GCT G	16S, 859	30	Glöckner et al. 2000
R-BT065	Cluster within beta I lineage of *Betaproteobacteria*	GTT GCC CCC TCT ACC GTT	16S, 65	55	Šimek et al. 2001
Bet2-870	Beta II lineage of *Betaproteobacteria*	CCC AGG CGG CTG ACT TCA	16S, 870	55	Burkert et al. 2003
MET1217	Beta IV lineage and relatives (*Betaproteobacteria*)	TTA CGT GTG AAG CCC TGG	16S, 1217	40	Friedrich et al. 2003
MET1217 C	Competitor for MET1217	TGA CGT GTG AAG CCC TGG	16S, 1217	40	Friedrich et al. 2003
H979	Helper 1 for MET1217	CAA GGC CAG GTA AGG	16S, 979	40	Friedrich et al. 2003
H1199	Helper 2 for MET1217	CCA TAA GGG CCA TGA GGA	16S, 1199	40	Friedrich et al. 2003
H1235	Helper 3 for MET1217	GTA CCG ACC ATW GTA	16S, 1235	40	Friedrich et al. 2003
PnecABD-445	Subclusters PnecA, B, D of beta II	GAG CTG CTG TTT CTT CCC	16S, 445	55	Hahn et al. 2005
PnecC-445	Subcluster PnecC	GAG CCG GTG TTT CTT CCC	16S, 445	55	Hahn et al. 2005
PnecD1-181	Part of subcluster PnecD	TTT CCC CCT AAG GGA TT	16S, 181	35	Hahn et al. 2005
PnecD2-181	Part of subcluster PnecD	TTT CCC CCT TAG GGA TT	16S, 181	35	Hahn et al. 2005
PnecA1-151	Part of subcluster PnecA	TTA CCG CAA CTT TCG CTG	16S, 151	35	Wu et al. 2006
PnecA2-151	Part of subcluster PnecA	TTA TCG CAA CTT TCG CTG	16S, 151	35	Wu et al. 2006
PnecB-23S-166	Subcluster PnecB	GTT CGC TCC ACA CAC CCT	23S, 166	60	Wu et al. 2006

OTHER PROTOCOLS

bacterial bulk activity

Triplicates: 5 ml of sample
Duplicates: 5 ml of prefixed sample

- Add 20 nM tritiated tracers (e.g. [^{3}H]-Thymidin, [^{3}H]-Leucine, [^{3}H]-amino acids,…)
- Incubate for 1-2 h @ *in situ* temperature in the dark
- Fix with 300 µl Formaldehyde (35%) or 500 µl of PFA (20%) for 1 h @ RT or over night @ +4°C
- Filter samples at low pressure (<200 mm Hg) onto nitrocellulose filters (0.2 µm pore size)
- Wash filters twice with 3 ml of ice-cold TCA (5%)
- Wash filters twice with 3 ml of ice-cold EtOH (80%)
- Let filters air dry, store @ -20°C
- Put filters in 5 ml scintillation vials
- Add 0.5 ml Ethyl acetate (filters must be completely covered)
- Let filters dissolve over night
- Vortex samples
- Add 4 ml of scintillation cocktail
- Vortex thoroughly
- Put samples in scintillation counter
- Start the appropriate program for [^{3}H]
- Wait for the output of dpms (disintegrations per minute)…
- Calculation of incorporation of radiolabeled tracers:

$$INC = \frac{U \times \left(4.5 \times 10^{-13}\right)}{SA \times t \times V}$$

where:
INC…incorporation of radiolabeled tracer (mmol l^{-1} h^{-1})
U…..dpm (mean of triplicates – controls)
4.5 x 10^{-13}…conversion factor (dpm to Ci)
SA….specific activity (Ci $mmol^{-1}$)
t …incubation time (h)
V…..Sample volume filtered (l)

- For thymidin incorporation, you can calculate newly produced cells, growth rates and doubling times:

$$Cells_{new} = mol^3H - THY \times 2 \times 10^{18}$$

where:
$Cells_{new}$...newly produced cells (cells l^{-1} h^{-1})
2 x 10^{18}…conversion factor

$$\mu = \frac{N_0 + Cells_{new}}{N_0}$$

where:
µ…bacterial growth rate
N_0…bacterial abundance

$$dt = \frac{\ln 2}{\mu}$$

where:
dt…bacterial doubling time

- For leucine incorporation, you can calculate total bacterial production:

$$BP = \frac{mol^3H - Leu \times 131.2}{0.073} \times 0.86 \times 2$$

where:
BP…….bacterial production (g C l^{-1} h^{-1})
131.2…molecular weight for leucine
0.073…%LEU (% leucine of all proteins)
0.86…..C/PROTEIN(cellular C:proteins)
2 ……..isotope dilution

MAR-FISH

Triplicates: 2-5 ml sample
1 prefixed control sample

- Add 20 nM tritiated tracers (e.g. [^{3}H]-Thymidin, [^{3}H]-Leucine, [^{3}H]-amino acids,…)
- Incubate for 2 h @ *in situ* temperature in the dark
- Fix with 500 µl of PFA (20%) for 1 h @ RT or over night @ +4°C
- Filter x ml sample onto white polycarbonate-filters (0.2 µm pore size + supporting filter, 0.45 µm pore size), rinse with MQ, let air dry, store @ -20°C
- **CARD-FISH…**
- Cut off one corner of a glass slide, clean it with EtOH
- Dip slide into molten 2% agarose and place hybridized filter sections with the bacteria facing upwards onto it (you have to work fast – agarose hardens quickly). Eight or even more filter sections can be placed on one slide

- Dry agarose @ ~40°C
- Glass slides with attached filter sections can be stored at -20°C
- Heat emulsion for 30-60 min in a water bath @ 43°C
- Get ice (or a precooled aluminium block), prepare boxes with silica gel & aluminium foil, cleaning tissues, and a forceps
- **Turn off the lights**
- Put the first slide in the emulsion, close the tube and gently agitate it for several seconds so that the whole slide is covered with emulsion
- Take out slide with forceps
- Clean back of the slide with a tissue
- Put slide for ~10 min at the aluminium block for hardening of the emulsion
- Get on with the next slide… with one aliquot of emulsion (20 ml), up to 20 slides can be processed at once
- Put slides in boxes, wrap them with aluminium foil
- **Turn on the lights**
- Expose slides for 2-4 days (depending on the activity status) @ +4°C (in the dark!!)

development of samples

- Fill up 4 containers with
  1. Developer (diluted 1:1 with MQ)
  2. MQ
  3. Fixer
  4. Tab water
- **Turn off the lights**
- Take slides out of the boxes & put them in a rack
- Put rack in developer for 2 min
- Put rack in MQ for 10 sec
- Put rack in fixer for 5 min
- Put rack in tab water for 5 min
- **Turn on the lights**
- Let slides air dry
- Embed slides in DAPI-MIX

material

Aluminium block (or ice)
Boxes (e.g. filter boxes)
Silica gel
Scintillation tubes or Falcon tubes
Storage tubes for glass slides
Glass slides & cover slips
Forceps
Tissues

solutions

2 % Agarose (200 ml)
4 g Agarose
200 ml MQ
Stir & heat until agarose is completely dissolved
Storage @ RT, melt it before use

Autoradiography emulsion
Dilute with 0.2 % agarose 1:1 (in the dark!)
Fill in storage tubes for slides (20 ml)
Wrap tubes with aluminium foil
Storage @ +4°C (several months)

0.2 % Agarose (200 ml)
0.4 g Agarose
200 ml MQ
Stir & heat until agarose is completely dissolved
Storage @ RT, melt it before use

ordering information

Kodak NTB autoradiography emulsion
Integra biosciences, www.integra-biosciences.de
IB05666

Dektol Developer
Sigma-Aldrich Co, www.sigmaaldrich.com
P6917-1GA

Kodak Fixer
Sigma-Aldrich Co, www.sigmaaldrich.com
P6557-1GA

Radiolabeled tracers
Amersham,
www.gehealthcare.com/amershamrads

Storage tubes for glass slides
Klösch Laborbedarf, Tel: 0043 01 4925185
10-00120

Nitrocellulose filters
Millipore
www.millipore.com
White, 25 mm diameter, 0.22 µm pore size: # GSWP02500

cloning & sequencing (16S rRNA)

- Prefilter 1l of unfixed sample over 8 µm polycarbonate filters
- Filter as much volume as possible onto 0.2 µm polycarbonate filters
- Store filters at -80°C

filter PCR:

- For 1 reaction you need:

10 µl 10x PCR buffer with $MgCl_2$
10 µl BSA (stock: 3 mg ml^{-1})
10 µl dNTPs (2.5 mM each)
0.5 µl Taq polymerase (2.5 U)
0.4 µl primer GM3F (stock: 50µM)
0.4 µl primer GM4R (stock: 50µM)
68.7 µl PCR-MQ
100 µl

- Use a small (~1mm^2) piece of biomass filter as template for the PCR

normal PCR with prior DNA isolation:

- use the power soil isolation kit (MOBIO), follow the instructions, except: elute DNA with 50 µl of PCR-MQ instead of solution C6
- For 1 reaction you need:

22.5 µl Platinum SuperMix High Fidelity
0.75 µl primer GM3F
0.75 µl primer GM4R
0.5-2 µl Template
~25 µl

- For a higher amount of product, make 5 replicate PCRs and pool them afterwards
- Cycle conditions:
  1. 94°C – 5 min
  2. 94°C – 1 min
  3. 48°C – 1.5 min
  4. 72°C – 2 min

  Go to 2 and repeat 28 times
  5. 72°C – 10 min
- Prepare agarose gel (0.8-1%) to check for ~1.5 kb products:

  4 µl 1kb ladder
  4 µl PCR product + 2 µl loading buffer
  75 V, ~30 min (small chamber)
  stain with EthBr for ~15-20 min
- Use the QIAquick PCR purification kit (follow the instructions, except: elute in 30 µl prewarmed PCR-MQ)

Alternatively you can also cut out bands from the gel (use wider combs, more PCR-product [8 µl] & two slots for each PCR-product) and use the QIAquick gel extraction kit instead…

- Measure DNA concentration

ligation & transformation:

- Prepare LB agar with Ampicillin, IPTG & X-Gal, 500 ml is enough for ~20 plates. Dry plates and prewarm them @ 37°C
- Use the TOPO TA cloning kit and follow the manufacturers' manual,
- Be very careful during ligation and transformation: do not mix reaction by pipeting up & down, do not vortex!
- Ligation can/should be prolongued to 30 min @ RT
- Spread 40-80 µl from each transformation onto triplicate prewarmed agar plates and incubate over night @ 37°C
- Pick WHITE clones with autoclaved toothpicks and transfer them to a master plate and 100 µl LB medium + ampicillin (use 96 well plates)
- Incubate over night @ 37°C
- For long-term storage of clone libraries, add glycerol (25% f.c.) and store @ -20°C (or -80°C)

screening of clone libraries:

- Check for inserts by PCR with primers M13F & M13R
- For 1 reaction you need:

1 µl 10x PCR buffer without $MgCl_2$
0.3 µl $MgCl_2$
0.2 µl dNTPs
0.05 µl Taq polymerase
0.3 µl primer M13f
0.3 µl primer M13r
1 µl template
6.85 µl PCR-MQ
10 µl

- As template use over night grown clones (in LB medium) which are repeatedly frozen and thawed to crack the cells
- Cycle conditions:
  1. 95°C – 4 min
  2. 95°C – 30 sec
  3. 60°C – 30 sec
  4. 72°C – 2 min

  Go to 2 and repeat 29 times
  5. 72°C – 10 min
- Prepare agarose gel (0.8%) to check for ~1.5 kb products
- Use the QIAprep spin Miniprep kit for preparation of vectors, follow the manufacturers protocol, except: elute DNA with 30 µl prewarmed (65°C) PCR-MQ, let stand for ~5 min and centrifuge afterwards

Measure DNA concentration

sequencing reaction:

- For 1 reaction you need:
  1 µl 2.5x buffer
  1 µl BigDye
  0.1 µl primer M13f (stock: 50µM)
  0.5 – 2 µl template (5-10 ng DNA)
  Add up to 5 µl with PCR-MQ
- Purification of sequencing reaction with Sephadex G-50 Superfine plates following the manufacturers instructions
- Ready for sequencing!!

solutions:

0.8% agarose gel
0.48 g agarose
60 ml 1 x TAE buffer

10 x TAE buffer
12.11 Tris (4 M)
13.61 g Na Actetat (1 M)
37.22 g EDTA (0.1 M)
1000 ml MQ
Adjust pH to 8

LB agar (~20 plates)
5 g Tryptone
2.5 g Yeast extract
5 g NaCl
7.5 g Agar
500 ml MQ
Autoclave, let cool down
Add: 100 µg ml-1 Ampicillin
0.5 mM IPTG
80 µg ml-1 X-Gal

5 µl

- Cycle conditions:
  1. 96°C – 10 sec
  2. 50°C – 5 sec
  3. 60°C – 4 min

  Go to 2 and repeat 39 times
  4. 21°C – 30 sec

1 x LB medium
10 g Tryptone
5 g Yeast extract
10 g NaCl
1000 ml MQ
Autoclave, let cool down
Add: 100 µg ml^{-1} Ampicillin

Ethidium bromide stock (1 mg ml-1)
0.05 g EthBr
50 ml MQ
Dilute to working solution (0.4 µg ml-1):
200 ml stock + 500 ml MQ

Primer sequences:
- GM3F (16S)
  5'-AGA GTT TGA TCM TGG C-3'
- GM4R (16S)
  5'-TAC CTT GTT ACG ACT T-3'
- M13F (vector)
  5'-GTA AAA CGA CGG CCA G-3'
- M13R (vector)
  5'-CAG GAA ACA GCT ATG AC-3
- GM1F (16S) – for full length sequences
  5'-CCA GCA GCC GCG GTA AT-3'

….and now for something completely different….

…acknowledgements…

Top-down pressure… needed from time to time???

supervisors (the big fish)

Thomas Posch	My dearest Tom, thanks for the ongoing friendship & support (scientific as well as emotional) – your presence & your (sometimes even funny) jokes make life (& science) worth living…
Jakob Pernthaler	Thank you for the warm welcome to your team, for help when help was needed (e.g. statistics, hard disc-crash, shortening of manuscripts…), for inspiring ideas & discussions, and for being a friend
Roland Psenner	Thanks for the instructive & inspirational time in Innsbruck & for discussions about Piburger See and all its inhabitants

Bottom-up effects… essential for growth

current colleagues (swiss universe dominated by non-swiss)

Michael Zeder	Dear mize-katze, without your mizoscope, your mizmobile, your mizprograms, your friendship & lots of other things I would be in a real fix… Thanks for being some kind of soulmate & keep on working in the lab! miau!
Judith Blom	Thanks for intense discussions about life, the universe & everything & especially for the cocktail-&-gossip-mondays! And don't forget: "Jungs – alle in einen Sack stecken…"
Eugen Loher	The most genius technician worldwide – thanks for those amazing self-made constructions that can make life much easier (e.g. the foot-mouse-counter, filtration devices) & for your sense of (swiss) humour
Markus Steinkellner	Thanks for help in the lab, for the nice time with the Sardinian starfish (esp. the underwater-sampling) & for preparing the best Birchermüesli ever
Astrid Kunz	Thank you for organizing at least a million things, for delicious cakes & for representing the soul of the institute
Tatiana Shabarova	Tanjushka, thanks for teaching me some nasty phrases in Russian, for the re-re-redesign of our aquarium, for debating about shitty PCR products & for continuous disturbance during intense writing periods ;-)
Karel Horňák	Thanks for teaching me nasty expressions in Czech, for introducing me to the (really) dark secrets of MAR & for being the only Czech I know who prefers whine, not beer
Silke van den Wyngart	Thanks for your enthusiasm about *Planktothrix* and stoichiometry - even if I understood just a small part; for your open, kind and inquisitive character & for long lasting support of the Tyrolean (Wildschönau) tourism
Ferdi Schanz	Thank you for your interesting lessons about Lake Zurich and basic limnology & for exciting excursions
Remo Freimann	Mr. FlowJo! Thanks for teaching me some swiss german (you have tried at least…) & of course for gruscheling me – can you now please explain me what exactly all these shitty DAPI+ EUB- debris particles are??
Heinz Maag	Without you I would not know how to make a proper palstek and other knots - thanks for the wonderful time in Sardinia (over and under water)
…& all the other limnols from ZH	Angela Mechsner, Thomas Ammann, Reto Bertschinger, Cecilia Alonso, Kasia Pivos - thanks for all the help, for funny jokes, delicious breakfasts & BBQ parties, coffee & cigarette breaks, interesting discussions…

former colleagues (tyrolean conquerors, vorarlbergean, spanish & south tyrolean invaders, an uruguayan mami & the czech mafia)

Pepi Franzoi	Thanks for the wonderful time we spent at Piburger See, all the chemical analyses, for your view of life & that you took (somehow) care of me
Bitzi Sonntag	Thanks for all the fun, the discussions, all the parties & drinks – and also for your enthusiasm about ciliates, your support during successful & unsuccessful experiments & for providing me a cosy bed whenever needed
Albin Alfreider	Dear Albinoni, thanks for all the hours we have spent in bars & restaurants ("ornithological excursions") with speaking about science while playing darts and getting drunk
Maite Pérez	Thanks for standing in with sampling (during my Bremen-trip), for discussing about FISH & MAR, and for your kind of humour – good luck with your not-jet-born daughter!
Martin Prader	Thanks for incredible U/W pictures & fascinating stories about diving with white sharks, whale sharks & mantas, and for providing fun in the lab
Silvia Knab	Thanks for being my office- & lab-mate and for being "Miss spring bloom"
…& all the other limnos from IBK	Ruben, Barbara, Karin, Paul, Hansjörg, Birgit S., Birgit M., Werner, Leo,… Thanks for the wonderful time in Innsbruck!!
Claudia Piccini	Thanks for help with cloning & sequencing, and for making the time in Bremen more colour- (and esp. music-)ful – all the best with little Falco!
Karel Šimek	Thanks for inspiring discussions, data interpretation & funny stories about professors - let all the butterflies be with you!
Jarda Vrba	Thanks for fruitful discussions about interactions of bacteria and protists while drinking incredible amounts of Budvar

the inner circle (family & friends)

my parents & my brother Tom	Thanks for mental and financial support during my study & for providing a place called "home" – even if it's quite far away
my friends	Matschi – what would I do without you? Thanks for the best friendship for so many years! Carole – thanks for numerous cinema-&-Caipirinha-Sessions in Innsbruck – this I really miss here! Thanks & a big hug to all my other friends!!!

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Lindt & Sprüngli, Nutella,…	…for energy supply (tons of chocolate & Nutella) & inspiration (gin tonic & parisienne) during intense periods of lab work, data evaluation & writing (i.e. always…)

…ein herzliches Vergeltsgott!
merci (mässi) vielmal!
thank you, danke, спасибо, gracias, danku, děkuji, dziękuję…

curriculum vitae

name Michaela Maria Salcher
born 16.01.1978 in Wörgl, Austria
nationality Austrian

education and research experience

1984–1988: Elementary school in Hopfgarten, Austria
1988–1992: Secondary school in Hopfgarten, Austria
1992–1998: HBLA in Kufstein, Austria

1998–2004: University of Innsbruck, Austria: Study of Biology (major subjects: Ecology and Limnology)

2003–2004: Diploma thesis at the Institute of Ecology (University of Innsbruck); Title: "Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community "; Supervisors: Dr. Thomas Posch, Univ.-Prof. Dr. Roland Psenner

10/2004–03/2006: Start of the PhD thesis at the Institute of Ecology (University of Innsbruck); Title: „Seasonality of bacterial phylotypes affected by bottom-up and top-down control in Piburger See, Austria"; Supervisors: Dr. Thomas Posch, Univ.-Prof. Dr. Roland Psenner; Employed as PhD student in the project FWF ZFP175540

Since 04/2006: Continuation of the PhD thesis at the Division of Limnology, Institute of Plant Biology, University of Zurich, Switzerland; Supervisors: Dr. Thomas Posch, Univ.-Prof. Dr. Jakob Pernthaler

award

17/12/2004: Appreciation award from the Austrian Ministry of Education, Science and Culture to the best Austrian University alumni for an outstanding diploma thesis

publications

Salcher MM, Psenner R, Pernthaler J, Posch T (2004) Es gibt nicht nur eine Antwort: Sukzession bakterieller Fraßschutzmechanismen, ausgelöst durch unterschiedliche Protistenarten. *Ber nat-med Ver Innsbruck, Supplementum* **15:**61.

Salcher MM, Psenner R, Pernthaler J, Posch T (2005) Succession of bacterial grazing defense mechanisms against protistan predation in an experimental microbial community. *Aquat Microb Ecol* **38:** 215-229.

Salcher MM, Hofer J, Horňàk K, Jezbera J, Sonntag B, Vrba J, Šimek K, Posch T (2007) Modulation of microbial predator-prey dynamics by phosphorus availability: Growth patterns and survival strategies of bacterial phylogenetic clades. *FEMS Microb Ecol* **60:** 40-50.

Posch T, Mindl B, Horňák K, Jezbera J, Salcher MM, Sattler B, Sonntag B, Vrba J, Šimek K (2007) Biomass reallocation within freshwater bacterioplankton induced by manipulating phosphorus availability and grazing. *Aquat Microb Ecol* **49:** 223-232.

Posch T, Franzoi J, Prader M, Salcher MM (submitted) Morphotype specific contributions of hybridized cells to total bacterioplankton abundance and biomass as studied by image analysis. Positive relation of cell size to hybridization efficiency? (submitted)

Salcher MM, Pernthaler J, Zeder M, Psenner R, Posch T (2008) Spatio-temporal niche differentiation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake (*Environ Microbiol* in press)

Salcher MM, Pernthaler J, Posch T (in prep) Abundance, biomass, or activity – which parameter mirrors best the dominance of freshwater bacterial clades?

presentations at workshops & meetings

Posch T, Šimek K, Jezbera J, Horňák K, Mindl B, Salcher MM, Sattler B, Sonntag B,Vrba J. Einfluss von Nährstoffen und Räuberdruck auf aquatische Bakterien – Dialyseschlauch-Experimente zur Analyse mikrobieller Interaktionen in situ (oral presentation). 25th Annual SIL-Austria Meeting, 28–30/10/2003, St. Georgen, Austria

Salcher MM, Pernthaler J, Psenner R, Posch T. Es gibt nicht nur eine Antwort – Sukzession bakterieller Fraßschutzmechanismen, ausgelöst durch unterschiedliche Protistenarten (oral presentation). 23th Annual Meeting of the German Society for Protozoology, 03–06/03/2004, Innsbruck, Austria

Salcher MM, Hofer J, Sonntag B, Simek K, Hornák K, Jezbera J, Vrba J, Mindl B, Sattler B, Posch T. Impact of flagellate grazing and nutrient availability on the taxonomic structure and activity of planktonic bacteria (poster presentation). 24th Annual Meeting of the German Society for Protozoology, 02–05/03/2005, Kaiserslautern, Germany

Posch T, Salcher MM. The critical nature of protistan test organisms: How to use the synergistic potential of studies on 'the same test organism'? (oral presentation). 24th Annual Meeting of the German Society for Protozoology, 02–05/03/2005, Kaiserslautern, Germany

Salcher MM, Pernthaler J, Psenner R, Posch T. Combining monitoring-based observations with experimental approaches to investigate bottom-up and top-down factors controlling natural freshwater bacterioplankton (poster presentation). Symposium Science and Art in Europe, 22–24/05/2005, Berlin and Jena, Germany

Salcher MM, Hofer J, Sonntag B, Simek K, Hornák K, Jezbera J, Vrba J, Mindl B, Sattler B, Posch T. Manipulation of protistan grazing pressure and phosphorus supply to elucidate the competitive abilities of eubacterial subgroups in a mesotrophic lake (poster presentation). ALSO-Summer Meeting, 19–24/06/2005, Santiago de Compostela, Spain

Posch T, Mindl B, Salcher MM, Sonntag B, Pernthaler J, Vrba J, Psenner R. Stoichiometry of bacteria, algae, and bacterivores: A reinvestigation of the 'phytoplankton-bacteria paradox' in a chemostat under different phosphorus supply. (oral presentation). ALSO-Summer Meeting, 19–24/06/2005, Santiago de Compostela, Spain

Salcher MM, Franzoi J, Psenner R, Posch T. towards a fine-scale analysis of bacterioplankton seasonality: Contrasting patterns of dominant bacterial clades in relation to biotic and abiotic factors (poster presentation). International Symposium on Microbial Ecology (ISME_11), 20-25/08/2006, Vienna, Austria

Salcher MM, Franzoi J, Pernthaler R, Posch T. Seasonal aspects of prominent bacterial clades and their response to bottom-up and top-down factors in an oligo-mesotrophic lake (poster presentation). Meeting of the Swiss Microbial Ecology (SME), 28-30/09/2006, Bellinzona, Switzerland

Posch T, Salcher MM, Pernthaler J, Psenner R. Recurrent patterns in bacterioplankton: predation pressure, nutrient limitation – or competition? (oral presentation). SIL-meeting, 12-18/08/2007, Montreal, Canada

Salcher MM, Pernthaler J, Posch T. Bacterial seasonality assessed on three levels: Abundance, biomass and activity of three phylogenetic clades (poster presentation). Meeting of PhD students of the Swiss Society of Hydrology and Limnology (SGHL), 16-17/08/2007, Piora, Switzerland

tutorials at students courses

Aquatic microbial ecology – 3 KP, SS 2004, University of Innsbruck, Austria
Aquatic microbial ecology – 6 KP, WS 2005/06, University of Zurich, Switzerland
Aquatic microbial ecology – 6 KP, WS 2006/07, University of Zurich, Switzerland
Aquatic microbial ecology – 6 KP, SS 2007, University of Zurich, Switzerland

hobbies

Music, books, water (swimming, diving)

Hell – is sitting on a hot stone reading your own scientific publications

Eric Ursin, fish biologist

(taken from: Sand-Jensen K (2007) How to write consistently boring scientific literature. *Oikos* 116: 723-727)

Hab nun, ach! die ~~Philosopheу~~, ⇐ Limnologie

~~Medizin~~ und ~~Juristereу~~ ⇐ Ökologie und Saisonalität von Bakterien

Und leider auch die ~~Theologie~~ ⇐ Molekularbiologie

Durchaus studirt mit heisser Müh.

Da steh ich nun, ich armer Thor,

Und binn so klug als wie zuvor.

J. W. Goethe, Faust Teil I, slightly modified…